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## PRIORITY DOCUMENT

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Docket No.  
1038-922 MIS:jb

TO THE ASSISTANT COMMISSIONER FOR PATENTS

Submitted herewith for filing under 35 U.S.C. 111 and 37 C.F.R. 1.53 is the patent application of:

Li, et al.

**NUCLEIC ACID RESPIRATORY SYNCYTIAL VIRUS VACCINES**

Enclosed are:

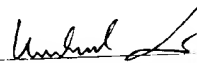
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- ☒ **Thirty-four (34)** sheets of drawings.
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**CLAIMS AS FILED**

For	#Filed	#Allowed	#Extra	Rate	Fee
Total Claims	54	- 20 =	34	x \$18.00	\$612.00
Indep. Claims	11	- 3 =	8	x \$78.00	\$624.00
Multiple Dependent Claims (check if applicable) <input type="checkbox"/>					\$0.00
BASIC FEE					\$760.00
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- ☒ A check in the amount of **\$1,996.00** to cover the filing fee is enclosed.
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Dated: March 4, 1999

  
Michael I. Stewart Signature (24,973)

CC:

TITLE OF INVENTION  
NUCLEIC ACID RESPIRATORY SYNCYTIAL VIRUS VACCINES

REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of  
5 copending United States Application No. 08/896,500 filed  
July 18, 1997, which itself is a continuation-in-part of  
copending United States Patent Application No.  
08/659,939 filed June 7, 1996 (now U.S. Patent No.  
5,843,913), which itself is continuation-in-part of  
10 copending United States Patent Application No.  
08/476,397, filed June 7, 1995.

FIELD OF INVENTION

The present invention is related to the field of  
Respiratory Syncytial Virus (RSV) vaccines and is  
15 particularly concerned with vaccines comprising nucleic  
acid sequences encoding the fusion (F) protein of RSV.

BACKGROUND OF INVENTION

Respiratory syncytial virus (RSV), a negative-  
strand RNA virus belonging to the *Paramyxoviridae* family  
20 of viruses, is the major viral pathogen responsible for  
bronchiolitis and pneumonia in infants and young  
children (ref. 1 - Throughout this application, various  
references are referred to in parenthesis to more fully  
describe the state of the art to which this invention  
25 pertains. Full bibliographic information for each  
citation is found at the end of the specification,  
immediately preceding the claims. The disclosures of  
these references are hereby incorporated by reference  
into the present disclosure). Acute respiratory tract  
30 infections caused by RSV result in approximately 90,000  
hospitalizations and 4,500 deaths per year in the United  
States (ref. 2). Medical care costs due to RSV  
infection are greater than \$340 M annually in the United  
States alone (ref. 3). There is currently no licensed  
35 vaccine against RSV. The main approaches for developing

an RSV vaccine have included inactivated virus, live-attenuated viruses and subunit vaccines.

The F protein of RSV is considered to be one of the most important protective antigens of the virus. There is a significant similarity (89% identity) in the amino acid sequences of the F proteins from RSV subgroups A and B (ref. 3) and anti-F antibodies can cross-neutralize viruses of both subgroups as well as protect immunized animals against infection with viruses from both subgroups (ref. 4). Furthermore, the F protein has been identified as a major target for RSV-specific cytotoxic T-lymphocytes in mice and humans (ref. 3 and ref. 5).

The use of RSV proteins as vaccines may have obstacles. Parenterally administered vaccine candidates have so far proven to be poorly immunogenic with regard to the induction of neutralizing antibodies in seronegative humans or chimpanzees. The serum antibody response induced by these antigens may be further diminished in the presence of passively acquired antibodies, such as the transplacentally acquired maternal antibodies which most young infants possess. A subunit vaccine candidate for RSV consisting of purified fusion glycoprotein from RSV infected cell cultures and purified by immunoaffinity or ion-exchange chromatography has been described (ref. 6). Parenteral immunization of seronegative or seropositive chimpanzees with this preparation was performed and three doses of 50 µg were required in seronegative animals to induce an RSV serum neutralizing titre of approximately 1:50. Upon subsequent challenge of these animals with wild-type RSV, no effect of immunization on virus shedding or clinical disease could be detected in the upper respiratory tract. The effect of immunization with this vaccine on virus shedding in the lower respiratory tract was not investigated, although this is the site where

the serum antibody induced by parenteral immunization may be expected to have its greatest effect. Safety and immunogenicity studies have been performed in a small number of seropositive individuals. The vaccine was found to be safe in seropositive children and in three seronegative children (all > 2.4 years of age). The effects of immunization on lower respiratory tract disease could not be determined because of the small number of children immunized. One immunizing dose in seropositive children induced a 4-fold increase in virus neutralizing antibody titres in 40 to 60% of the vaccinees. Thus, insufficient information is available from these small studies to evaluate the efficacy of this vaccine against RSV-induced disease. A further problem facing subunit RSV vaccines is the possibility that inoculation of seronegative subjects with immunogenic preparations might result in disease enhancement (sometimes referred to as immunopotential), similar to that seen in formalin inactivated RSV vaccines. In some studies, the immune response to immunization with RSV F protein or a synthetic RSV FG fusion protein resulted in a disease enhancement in rodents resembling that induced by a formalin-inactivated RSV vaccine. The association of immunization with disease enhancement using non-replicating antigens suggests caution in their use as vaccines in seronegative humans.

Live attenuated vaccines against disease caused by RSV may be promising for two main reasons. Firstly, infection by a live vaccine virus induces a balanced immune response comprising mucosal and serum antibodies and cytotoxic T-lymphocytes. Secondly, infection of infants with live attenuated vaccine candidates or naturally acquired wild-type virus is not associated with enhanced disease upon subsequent natural reinfection. It will be challenging to produce live

attenuated vaccines that are immunogenic for younger infants who possess maternal virus-neutralizing antibodies and yet are attenuated for seronegative infants greater than or equal to 6 months of age.

- 5 Attenuated live virus vaccines also have the risks of residual virulence and genetic instability.

Injection of plasmid DNA containing sequences encoding a foreign protein has been shown to result in expression of the foreign protein and the induction of  
10 antibody and cytotoxic T-lymphocyte responses to the antigen in a number of studies (see, for example, refs. 7, 8, 9). The use of plasmid DNA inoculation to express viral proteins for the purpose of immunization may offer several advantages over the strategies summarized above.  
15 Firstly, DNA encoding a viral antigen can be introduced in the presence of antibody to the virus itself, without loss of potency due to neutralization of virus by the antibodies. Secondly, the antigen expressed *in vivo* should exhibit a native conformation and, therefore,  
20 should induce an antibody response similar to that induced by the antigen present in the wild-type virus infection. In contrast, some processes used in purification of proteins can induce conformational changes which may result in the loss of immunogenicity  
25 of protective epitopes and possibly immunopotentiality. Thirdly, the expression of proteins from injected plasmid DNAs can be detected *in vivo* for a considerably longer period of time than that in virus-infected cells, and this has the theoretical advantage of prolonged  
30 cytotoxic T-cell induction and enhanced antibody responses. Fourthly, *in vivo* expression of antigen may provide protection without the need for an extrinsic adjuvant.

The ability to immunize against disease caused by  
35 RSV by administration of a DNA molecule encoding an RSV F protein was unknown before the present invention. In

particular, the efficacy of immunization against RSV induced disease using a gene encoding a secreted form of the RSV F protein was unknown. Infection with RSV leads to serious disease. It would be useful and desirable to provide isolated genes encoding RSV F protein and vectors for *in vivo* administration for use in immunogenic preparations, including vaccines, for protection against disease caused by RSV and for the generation of diagnostic reagents and kits. In particular, it would be desirable to provide vaccines that are immunogenic and protective in humans, including seronegative infants, that do not cause disease enhancement (immunopotential).

#### SUMMARY OF INVENTION

The present invention relates to a method of immunizing a host against disease caused by respiratory syncytial virus, to nucleic acid molecules used therein, and to diagnostic procedures utilizing the nucleic acid molecules. In particular, the present invention is directed towards the provision of nucleic acid respiratory syncytial virus vaccines.

In accordance with one aspect of the invention, there is provided an immunogenic composition for *in vivo* administration to a host for the generation in the host of a protective immune response to RSV F protein, comprising a non-replicating vector comprising:

a first nucleotide sequence encoding an RSV F protein or a RSV F protein fragment that generates antibodies and/or cytotoxic T-lymphocytes (CTLs) that specifically react with RSV F protein;

a promoter sequence operatively coupled to the first nucleotide sequence for expression of the RSV F protein, and

a second nucleotide sequence located adjacent the first nucleotide sequence to enhance the

immunoprotective ability of the RSV F protein when expressed in vivo from the vector in a host; and a pharmaceutically-acceptable carrier therefor.

The first nucleotide sequence may be that which encodes a full-length RSV F protein, as seen in Figure 2 (SEQ ID No: 2). Alternatively, the first nucleotide sequence may be that which encodes an RSV F protein from which the transmembrane region is absent. The latter embodiment may be provided by a nucleotide sequence which encodes a full-length RSV F protein but contains a translational stop codon immediately upstream of the start of the transmembrane coding region, thereby preventing expression of a transmembrane region of the RSV F protein, as seen in Figure 3 (SEQ. ID No. 4). The lack of expression of the transmembrane region results in a secreted form of the RSV F protein.

The first nucleotide sequence may encode a RSV F protein fragment lacking an autologous RSV F signal peptide sequence and may include, in its place, a sequence encoding a heterologous signal peptide sequence which enhances the level of expression of the RSV F protein. One signal peptide which has been found useful in this regard is the signal peptide of Herpes Simplex Virus I (HSV I)gD. Such enhanced expression levels also lead to improve immunogenicity of the vector at the same dosage level. The first nucleotide sequence may also encode a RSV F protein fragment lacking a transmembrane coding region.

The second nucleotide sequence may comprise a pair of splice sites to prevent aberrant mRNA splicing, whereby substantially all transcribed mRNA encodes the RSV protein. Such second nucleotide sequence may be located between the first nucleotide sequence and the promoter sequence. Such second nucleotide sequence may be that of rabbit  $\beta$ -globin intron II, as shown in Figure 8 (SEQ ID No: 5).



A vector encoding the F protein and provided by this aspect of the invention may specifically be pXL2 or pXL4 or p82M35B, as seen in Figures 5, 7 or 10, respectively.

5 The promoter sequence may be an immediate early cytomegalovirus (CMV) promoter.

Certain of the vectors provided herein may be used to immunize a host against RSV infection or disease by in vivo expression of RSV F protein lacking a  
10 transmembrane region following administration of the vectors. In accordance with a further aspect of the present invention, therefore, there is provided a method of immunizing a host against disease caused by infection with respiratory syncytial virus, which comprises  
15 administering to the host an effective amount of a non-replicating vector comprising a first nucleotide sequence encoding an RSV F protein or a RSV F protein fragment that generates antibodies and/or CTLs that specifically react with RSV F protein and a promoter  
20 sequence operatively coupled to the first nucleotide sequence for expression of the RSV F protein in the host, which may be a human. The promoter may be an immediate early cytomegalovirus promoter.

The nucleotide sequence may encode a truncated RSV  
25 F protein lacking the transmembrane region may be that as described above and/or possess a heterologous signal peptide encoding sequence.

The vector may contain a second nucleotide sequence located adjacent a first nucleotide sequence and  
30 effective to enhance the immunoprotective ability of the RSV F protein expressed by the first nucleotide sequence may be used to immunize a host. Specific non-replicating vectors which may be used in this aspect of the invention are those identified as plasmid vectors  
35 pXL2, pXL4 and p82M35B in Figures 5, 7 and 10 respectively.

The present invention also includes a novel method of using a gene encoding an RSV F protein or a RSV F protein fragment that generates antibodies and/or CTLs that specifically react with RSV F protein to protect a host against disease caused by infection with respiratory syncytial virus, which comprises:

isolating the gene;

operatively linking the gene to at least one control sequence to produce a non-replicating vector, said control sequence directing expression of the RSV F protein when said vector is introduced into a host to produce an immune response to the RSV F protein or fragment thereof, and

introducing the vector into the host.

The procedure provided in accordance with this aspect of the invention may further include the step of:

operatively linking the gene to an immunoprotection enhancing sequence to produce an enhanced immunoprotection by the RSV F protein in the host, preferably by introducing the immunoprotection enhancing sequence between the control sequence and the gene.

In addition, the present invention includes a method of producing a vaccine for protection of a host against disease caused by infection with respiratory syncytial virus, which comprises:

isolating a first nucleotide sequence encoding an RSV F protein or a RSV F protein fragment that generates antibodies and/or CTLs that specifically react with RSV F protein;

operatively linking the first nucleotide sequence to at least one control sequence to produce a non-replicating vector, the control sequence directing expression of the RSV F protein when introduced into a host to produce an immune response to the RSV F protein when expressed *in vivo* from the vector in a host, and

formulating the vector as a vaccine for in vivo administration.

The first nucleotide sequence further may be operatively linked to a second nucleotide sequence to enhance the immunoprotective ability of the RSV F protein when expressed in vivo from the vector in a host. The vector may be a plasmid vector selected from pXL2, pXL4 and p82M35B. The invention further includes a vaccine for administration to a host, including a human host, produced by this method as well as immunogenic compositions comprising an immunoeffective amount of the vectors described herein.

As noted previously, the vectors provided herein are useful in diagnostic applications. In a further aspect of the invention, therefore, there is provided a method of determining the presence of an RSV F protein in a sample, comprising the steps of:

- (a) immunizing a host with a non-replicating vector comprising a first nucleotide sequence encoding an RSV F protein or a RSV F protein fragment that generates antibodies and/or cytotoxic T-lymphocytes (CTLs) that specifically react with RSV F protein and a promoter sequence operatively coupled to the first nucleotide sequence for expression of the RSV F protein in the host to produce antibodies specific for the RSV F protein;
- (b) isolating the RSV F protein specific antibodies;
- (c) contacting the sample with the isolated antibodies to produce complexes comprising any RSV F protein present in the sample and the RSV F protein-specific antibodies; and
- (d) determining production of the complexes.

The non-replicating vector employed to elicit the antibodies may be a plasmid vector which is pXL1, pXL2, pXL3, pXL4 or p82M35B.

The invention also includes a diagnostic kit for detecting the presence of an RSV F protein in a sample, comprising:

- 5 (a) a non-replicating vector comprising a first nucleotide sequence encoding an RSV F protein or a RSV F protein fragment that generates antibodies that specifically react with RSV F protein and a promoter sequence operatively coupled to said first nucleotide sequence for expression of said RSV F  
10 protein in a host immunized therewith to produce antibodies specific for the RSV F protein;
- (b) isolation means to isolate said RSV F protein specific antibodies;
- (c) contacting means to contact the isolated RSV  
15 F specific antibodies with the sample to produce a complex comprising any RSV F protein present in the sample and RSV F protein specific antibodies; and
- (d) identifying means to determine production of the complex.

- 20 The present invention is further directed to a method for producing RSV F protein specific polyclonal antibodies comprising the use of the immunization method described herein, and further comprising the step of isolating the RSV F protein specific polyclonal  
25 antibodies from the immunized animal.

The present invention is also directed to a method for producing monoclonal antibodies specific for an F protein of RSV, comprising the steps of:

- 30 (a) constructing a non-replicating vector comprising a first nucleotide sequence encoding a RSV F protein and a promoter sequence operatively coupled to said first nucleotide sequence for expression of said RSV F protein; and, optionally,  
35 a second nucleotide sequence located adjacent said first nucleotide sequence to enhance the

immunoprotective ability of said RSV F protein when expressed *in vivo* from said vector in a host.

- (b) administering the vector to at least one mouse to produce at least one immunized mouse;
- 5 (c) removing B-lymphocytes from the at least one immunized mouse;
- (d) fusing the B-lymphocytes from the at least one immunized mouse with myeloma cells, thereby producing hybridomas;
- 10 (e) cloning the hybridomas;
- (f) selecting clones which produce anti-F protein antibody;
- (g) culturing the anti-F protein antibody-producing clones; and
- 15 (h) isolating anti-F protein monoclonal antibodies.

In this application, the term "RSV F protein" is used to define (1) a full-length RSV F protein, such proteins having variations in their amino acid sequences including those naturally occurring in various strains  
20 of RSV, (2) a secreted form of RSV F protein lacking a transmembrane region, and (3) functional analogs of the RSV F protein. In this application, a first protein is a "functional analog" of a second protein if the first  
25 protein is immunologically related to and/or has the same function as the second protein. The functional analog may be, for example, a fragment of the protein or a substitution, addition or deletion mutant thereof. Included are RSV F protein fragments that generate  
30 antibodies and/or CTLs that specifically react with RSV F protein.

#### BRIEF DESCRIPTION OF THE FIGURES

The present invention will be further understood from the following General Description and Examples with  
35 reference to the Figures in which:

Figure 1 illustrates a restriction map of the gene encoding the F protein of Respiratory Syncytial Virus;

Figures 2A, 2B, 2C, 2D and 2E show the nucleotide sequence of the gene encoding the membrane attached form of the F protein of Respiratory Syncytial Virus (SEQ ID No: 1) as well as the amino acid sequence of the RSV F protein encoded thereby (SEQ ID No: 2);

Figures 3A, 3B, 3C and 3D show the nucleotide sequence of the gene encoding the secreted form of the RSV F protein lacking the transmembrane region (SEQ ID No: 3) as well as the amino acid sequence of the truncated RSV F protein lacking the transmembrane region encoded thereby (SEQ ID No: 4);

Figures 4A, 4B, 4C and 4D show the construction of plasmid pXL1 containing the gene encoding a secreted form of the RSV F protein lacking the transmembrane region;

Figures 5A, 5B, 5C and 5D show the construction of plasmid pXL2 containing a gene encoding a secreted form of the RSV F protein lacking the transmembrane region and containing the rabbit  $\beta$ -globin Intron II sequence;

Figures 6A, 6B, 6C and 6D show the construction of plasmid pXL3 containing the gene encoding a full length membrane attached form of the RSV F protein;

Figure 7 shows the construction of plasmid pXL4 containing a gene encoding a membrane attached form of the RSV F protein and containing the rabbit  $\beta$ -globin Intron II sequence;

Figure 8 shows the nucleotide sequence for the rabbit  $\beta$ -globin Intron II sequence (SEQ ID No. 5);

Figure 9 shows the lung cytokine expression profile in DNA-immunized mice after RSV challenge;

Figure 10 is a schmmatic showing the assembly of plasmid p82M35B containing a gene encoding a secreted form of the RSV F protein lacking the transmembrane

region, the rabbit  $\beta$ -globin Intron II sequence and the signal peptide sequence HSV I gD;

Figure 11 shows the nucleotide sequence of plasmid VR-1012 (SEQ ID No: 6); and

5        Figure 12 shows DNA (SEQ ID No: 7) and derived amino acid (SEQ ID No: 8) sequence of the HSV gD signal peptide sequence, synthesized as a synthetic oligopeptide.

#### GENERAL DESCRIPTION OF INVENTION

10        As described above, the present invention relates generally to polynucleotide, including DNA, immunization to obtain protection against infection by respiratory syncytial virus (RSV) and to diagnostic procedures using particular vectors. In the present invention, several  
15 recombinant vectors were constructed to contain a nucleotide sequence encoding an RSV F protein.

The nucleotide sequence of the full length RSV F gene is shown in Figure 2 (SEQ ID No: 1). Certain constructs provided herein include the nucleotide  
20 sequence encoding the full-length RSV F (SEQ ID No: 2) protein while others include an RSV F gene modified by insertion of termination codons immediately upstream of the transmembrane coding region (see Figure 3, SEQ ID No: 3), to prevent expression of the transmembrane  
25 portion of the protein and to produce a secreted or truncated RSV F protein lacking a transmembrane region (SEQ ID No. 4). In addition, certain constructs provided herein include a nucleic acid sequence encoding a heterologous signal peptide sequence rather than the  
30 native signal peptide sequence to provide for enhanced protein expression and increased immunogenicity. Specifically, the signal peptide sequence for HSV I gD is employed. However, other heterologous signal peptides may be employed, such as that of human tissue  
35 plasminogen activator (TPA).

The nucleotide sequence encoding the RSV F protein is operatively coupled to a promoter sequence for expression of the encoded RSV F protein. The promoter sequence may be the immediately early cytomegalovirus (CMV) promoter. This promoter is described in ref. 13.

Any other convenient promoter may be used, including constitutive promoters, such as, Rous Sarcoma Virus LTRs, and inducible promoters, such as metallothionine promoter, and tissue specific promoters.

The vectors provided herein, when administered to an animal, effect *in vivo* RSV F protein expression, as demonstrated by an antibody response in the animal to which it is administered. Such antibodies may be used herein in the detection of RSV protein in a sample, as described in more detail below. When the encoded RSV F protein is in the form of an RSV F protein from which the transmembrane region is absent, such as plasmid pXL1 (Figure 4), the administration of the vector conferred protection in mice and cotton rats to challenge by live RSV virus neutralizing antibody and cell mediated immune responses and an absence of immunopotential in immunized animals, as seen from the Examples below.

The recombinant vector also may include a second nucleotide sequence located adjacent the RSV F protein encoding nucleotide sequence to enhance the immunoprotective ability of the RSV F protein when expressed *in vivo* in a host. Such enhancement may be provided by increased *in vivo* expression, for example, by increased mRNA stability, enhanced transcription and/or translation. This additional sequence preferably is located between the promoter sequence and the RSV F protein-encoding sequence.

This enhancement sequence may comprise a pair of splice sites to prevent aberrant mRNA splicing during transcription and translation so that substantially all transcribed mRNA encodes an RSV F protein.



Specifically, the rabbit  $\beta$ -globin Intron II sequence shown in Figure 7 (SEQ ID No: 5) may provide such splice sites, as also described in ref. 15.

The construct containing the Intron II sequence, 5 CMV promoter and nucleotide sequence coding for the truncated RSV F protein lacking a transmembrane region, i.e. plasmid pXL2 (Figure 5), induced complete protection in mice against challenge with live RSV, as seen in the Examples below. In addition, the construct 10 containing the Intron II sequence, CMV promoter and nucleotide sequence coding for the full-length RSV F protein, i.e. plasmid pXL4 (Figure 7), also conferred protection in mice to challenge with live RSV, as seen from the Examples below. The construct containing the 15 Intron II sequence, CMV promoter, HSV I gD signal peptide peptide encoding sequence and nucleotide sequence coding for the truncated RSV F protein lacking a transmembrane region, i.e. plasmid p82M35B (Figure 10), induced complete protection in the absence of 20 cardotoxin pretreatment under conditions where pretreatment with cardiotoxin was required for pXL2 to confer complete protection, as seen from the Examples below.

The vector provided herein may also comprise a 25 third nucleotide sequence encoding a further antigen from RSV, an antigen from at least one other pathogen or at least one immunomodulating agent, such as cytokine. Such vector may contain said third nucleotide sequence in a chimeric or a bicistronic structure. Alternatively, 30 vectors containing the third nucleotide sequence may be separately constructed and coadministered to a host, with the nucleic acid molecule provided herein.

It is clearly apparent to one skilled in the art, that the various embodiments of the present invention 35 have many applications in the fields of vaccination, diagnosis and treatment of RSV infections. A further

non-limiting discussion of such uses is further presented below.

#### 1. Vaccine Preparation and Use

Immunogenic compositions, suitable to be used as vaccines, may be prepared from the RSV F genes and vectors as disclosed herein. The vaccine elicits an immune response in a subject which includes the production of anti-F antibodies. Immunogenic compositions, including vaccines, containing the nucleic acid may be prepared as injectables, in physiologically-acceptable liquid solutions or emulsions for polynucleotide administration. The nucleic acid may be associated with liposomes, such as lecithin liposomes or other liposomes known in the art, as a nucleic acid liposome (for example, as described in WO 9324640, ref. 17) or the nucleic acid may be associated with an adjuvant, as described in more detail below. Liposomes comprising cationic lipids interact spontaneously and rapidly with polyanions such as DNA and RNA, resulting in liposome/nucleic acid complexes that capture up to 100% of the polynucleotide. In addition, the polycationic complexes fuse with cell membranes, resulting in an intracellular delivery of polynucleotide that bypasses the degradative enzymes of the lysosomal compartment. Published PCT application WO 94/27435 describes compositions for genetic immunization comprising cationic lipids and polynucleotides. Agents which assist in the cellular uptake of nucleic acid, such as calcium ions, viral proteins and other transfection facilitating agents, may advantageously be used.

Polynucleotide immunogenic preparations may also be formulated as microcapsules, including biodegradable time-release particles. Thus, U.S. Patent 5,151,264 describes a particulate carrier of a phospholipid/glycolipid/polysaccharide nature that has

been termed Bio Vecteurs Supra Moléculaires (BVSM). The particulate carriers are intended to transport a variety of molecules having biological activity in one of the layers thereof.

- 5 U.S. Patent 5,075,109 describes encapsulation of the antigens trinitrophenylated keyhole limpet hemocyanin and staphylococcal enterotoxin B in 50:50 poly (DL-lactide-co-glycolide). Other polymers for encapsulation are suggested, such as poly(glycolide),  
 10 poly(DL-lactide-co-glycolide), copolyoxalates, polycaprolactone, poly(lactide-co-caprolactone), poly(esteramides), polyorthoesters and poly(8-hydroxybutyric acid), and polyanhydrides.

- Published PCT application WO 91/06282 describes a  
 15 delivery vehicle comprising a plurality of bioadhesive microspheres and antigens. The microspheres being of starch, gelatin, dextran, collagen or albumin. This delivery vehicle is particularly intended for the uptake of vaccine across the nasal mucosa. The delivery  
 20 vehicle may additionally contain an absorption enhancer.

- The RSV F genes and vectors may be mixed with pharmaceutically acceptable excipients which are compatible therewith. Such excipients may include, water, saline, dextrose, glycerol, ethanol, and  
 25 combinations thereof. The immunogenic compositions and vaccines may further contain auxiliary substances, such as wetting or emulsifying agents, pH buffering agents, or adjuvants to enhance the effectiveness thereof. Immunogenic compositions and vaccines may be  
 30 administered parenterally, by injection subcutaneously, intravenously, intradermally or intramuscularly, possibly following pretreatment of the injection site with a local anesthetic. Alternatively, the immunogenic compositions formed according to the present invention,  
 35 may be formulated and delivered in a manner to evoke an immune response at mucosal surfaces. Thus, the

immunogenic composition may be administered to mucosal surfaces by, for example, the nasal or oral (intragastric) routes. Alternatively, other modes of administration including suppositories and oral formulations may be desirable. For suppositories, binders and carriers may include, for example, polyalkalene glycols or triglycerides. Oral formulations may include normally employed incipients, such as, for example, pharmaceutical grades of saccharine, cellulose and magnesium carbonate.

The immunogenic preparations and vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective, protective and immunogenic.

15 The quantity to be administered depends on the subject to be treated, including, for example, the capacity of the individual's immune system to synthesize the RSV F protein and antibodies thereto, and if needed, to produce a cell-mediated immune response. Precise

20 amounts of active ingredient required to be administered depend on the judgment of the practitioner. However, suitable dosage ranges are readily determinable by one skilled in the art and may be of the order of about 1  $\mu$ g to about 1 mg of the RSV F genes and vectors. Suitable

25 regimes for initial administration and booster doses are also variable, but may include an initial administration followed by subsequent administrations. The dosage may also depend on the route of administration and will vary according to the size of the host. A vaccine which

30 protects against only one pathogen is a monovalent vaccine. Vaccines which contain antigenic material of several pathogens are combined vaccines and also belong to the present invention. Such combined vaccines contain, for example, material from various pathogens or

35 from various strains of the same pathogen, or from combinations of various pathogens.

Immunogenicity can be significantly improved if the vectors are co-administered with adjuvants, commonly used as 0.05 to 0.1 percent solution in phosphate-buffered saline. Adjuvants enhance the immunogenicity of an antigen but are not necessarily immunogenic themselves. Adjuvants may act by retaining the antigen locally near the site of administration to produce a depot effect facilitating a slow, sustained release of antigen to cells of the immune system. Adjuvants can also attract cells of the immune system to an antigen depot and stimulate such cells to elicit immune responses.

Immunostimulatory agents or adjuvants have been used for many years to improve the host immune responses to, for example, vaccines. Thus, adjuvants have been identified that enhance the immune response to antigens. Some of these adjuvants are toxic, however, and can cause undesirable side-effects, making them unsuitable for use in humans and many animals. Indeed, only aluminum hydroxide and aluminum phosphate (collectively commonly referred to as alum) are routinely used as adjuvants in human and veterinary vaccines.

A wide range of extrinsic adjuvants and other immunomodulating material can provoke potent immune responses to antigens. These include saponins complexed to membrane protein antigens to produce immune stimulating complexes (ISCOMS), pluronic polymers with mineral oil, killed mycobacteria in mineral oil, Freund's complete adjuvant, bacterial products, such as muramyl dipeptide (MDP) and lipopolysaccharide (LPS), as well as monophoryl lipid A, QS 21 and polyphosphazene.

In particular embodiments of the present invention, the vector comprising a first nucleotide sequence encoding an F protein of RSV may be delivered in conjunction with a targeting molecule to target the

vector to selected cells including cells of the immune system.

The polynucleotide may be delivered to the host by a variety of procedures, for example, Tang et al. (ref. 5 10) disclosed that introduction of gold microprojectiles coated with DNA encoding bovine growth hormone (BGH) into the skin of mice resulted in production of anti-BGH antibodies in the mice, while Furth et al. (ref. 11) showed that a jet injector could be used to transfect 10 skin, muscle, fat and mammary tissues of living animals.

## 2. Immunoassays

The RSV F genes and vectors of the present invention are useful as immunogens for the generation of anti-F antibodies for use in immunoassays, including 15 enzyme-linked immunosorbent assays (ELISA), RIAs and other non-enzyme linked antibody binding assays or procedures known in the art. In ELISA assays, the vector first is administered to a host to generate antibodies specific to the RSV F protein. These RSV F- 20 specific antibodies are immobilized onto a selected surface, for example, a surface capable of binding the antibodies, such as the wells of a polystyrene microtiter plate. After washing to remove incompletely adsorbed antibodies, a nonspecific protein such as a 25 solution of bovine serum albumin (BSA) that is known to be antigenically neutral with regard to the test sample may be bound to the selected surface. This allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background 30 caused by nonspecific bindings of antisera onto the surface.

The immobilizing surface is then contacted with a sample, such as clinical or biological materials, to be tested in a manner conducive to immune complex 35 (antigen/antibody) formation. This procedure may include diluting the sample with diluents, such as

solutions of BSA, bovine gamma globulin (BGG) and/or phosphate buffered saline (PBS)/Tween. The sample is then allowed to incubate for from about 2 to 4 hours, at temperatures such as of the order of about 20° to 37°C.

- 5 Following incubation, the sample-contacted surface is washed to remove non-immunocomplexed material. The washing procedure may include washing with a solution, such as PBS/Tween or a borate buffer. Following formation of specific immunocomplexes between the test  
10 sample and the bound RSV F specific antibodies, and subsequent washing, the occurrence, and even amount, of immunocomplex formation may be determined.

#### BIOLOGICAL MATERIALS

- Certain plasmids that contain the gene encoding RSV  
15 F protein and referred to herein have been deposited with the America Type Culture Collection (ATCC) located at 10801 University Blvd., Manassas, VA 20110-2209, U.S.A., pursuant to the Budapest Treaty and prior to the filing of this application.

- 20 Samples of the deposited plasmids will become available to the public upon grant of a patent based upon this United States patent application and all restrictions on access to the deposits will be removed at that time. The deposits will be replaced if the  
25 Depository is unable to dispense viable samples. The invention described and claimed herein is not to be limited in scope by plasmids deposited, since the deposited embodiment is intended only as an illustration of the invention. Any equivalent or similar plasmids  
30 that encode similar or equivalent antigens as described in this application are within the scope of the invention.

	<u>Plasmid</u>	<u>ATCC Designation</u>	<u>Date Deposited</u>
	pXL1	97167	May 30, 1995
	pXL2	97168	May 30, 1995
	pXL3	97169	May 30, 1995
5	pXL4	97170	May 30, 1995
	p82M35B		

### EXAMPLES

The above disclosure generally describes the present invention. A more complete understanding can be  
 10 obtained by reference to the following specific Examples. These Examples are described solely for purposes of illustration and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as  
 15 circumstances may suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitations.

Methods of molecular genetics, protein  
 20 biochemistry, and immunology used but not explicitly described in this disclosure and these Examples are amply reported in the scientific literature and are well within the ability of those skilled in the art.

#### Example 1

25 This Example describes the construction of vectors containing the RSV F gene.

Figure 1 shows a restriction map of the gene encoding the F protein of Respiratory Syncytial Virus and Figure 2 shows the nucleotide sequence of the gene  
 30 encoding the full-length RSV F protein (SEQ ID No: 1) and the deduced amino acid sequence (SEQ ID No: 2). Figure 3 shows the gene encoding the secreted RSV F protein (SEQ ID No: 3) and the deduced amino acid sequence (SEQ ID No: 4).

35 A set of four plasmid DNA constructs were made (as shown schematically in Figures 4 to 7) in which cDNA



encoding the RSV-F was subcloned downstream of the immediate-early promoter, enhancer and intron A sequences of human cytomegalovirus (CMV) and upstream of the bovine growth hormone (BGH) poly-A site. The 1.6  
5 Kb Sspl-PstI fragment containing the promoter, enhancer and intron A sequences of CMV Towne strain were initially derived from plasmid pRL43a obtained from Dr. G.S. Hayward of Johns Hopkins University (ref. 20) and subcloned between EcoRV and PstI sites of pBluescript 11  
10 SK +/- (Stratagene). For the construction of plasmids expressing the secretory form of the F protein (pXL1 and pXL2 in Figs. 4 and 5), the 1.6 Kb EcoRI-BamHI fragment containing the truncated form of the F cDNA originally cloned from a clinical isolate belonging to subgroup A  
15 was excised from pRSVF (ref. 18 and WO 93/14207) and subcloned between EcoRI and BamHI sites of pSG5 (Stratagene, ref. 14). Either the 1.6 kb EcoRI-BamHI fragment or the 2.2 kb ClaI-BamHI fragment was then excised from the pSG5 construct, filled-in with Klenow  
20 and subcloned at the SmaI site of the pBluescript II SK +/- construct containing the promoter and intron A sequences. The 0.6 kb ClaI-EcoRI fragment derived from pSG5 contained the intron II sequences from rabbit  $\beta$ -globin. Subsequently, the plasmids were digested with  
25 HindIII, filled-in with Klenow, and digested with XbaI to yield either a 3.2 or a 3.8 Kb fragment. These fragments were used to replace the 0.8 kb NruI-XbaI fragment containing the CMV promoter in pRc/CMV (Invitrogen), resulting in the final pXL1 and pXL2  
30 constructs, respectively.

For the construction of plasmids expressing the full-length F protein (pXL3 and pXL4 - Figs. 6 and 7), the full length RSV F cDNA was excised as a 1.9 kb EcoRI fragment from a recombinant pBluescript M13-SK  
35 (Stratagene) containing the insert (ref. 18 and WO 93/14207) and subcloned at the EcoRI site of pSG5

(Stratagene). Either the 1.9 Kb EcoRI fragment or the 2.5 Kb ClaI-BamHI fragment was then excised from the pSG5 construct, filled-in with Klenow and subcloned at the SmaI site of the pBluescript II SK +/- construct containing the promoter and intron A sequences. The rest of the construction for pXL3 and pXL4 was identical to that for pXL1 and pXL2, as described above. Therefore, except for the CMV promoter and intron A sequences, the rest of the vector components in pXL1-4 were derived from plasmid pRc/CMV. Plasmids pXL1 and pXL2 were made to express a truncated/secretory form of the F protein which carried stop codons resulting in a C-terminal deletion of 48 amino acids including the transmembrane (TM) and the C-terminal cytosolic tail as compared to the intact molecule. In contrast, pXL3 and pXL4 were made to express the intact membrane-attached form of the RSV F molecule containing the TM and the cytosolic C-terminal tail. The rationale for the presence of the intron II sequences in pXL2 and pXL4 was that this intron was reported to mediate the correct splicing of RNAs. Since mRNA for the RSV-F has been suspected to have a tendency towards aberrant splicing, the presence of the intron II sequences might help to overcome this. All four plasmid constructs were confirmed by DNA sequencing analysis.

Plasmid DNA was purified using plasmid mega kits from Qiagen (Chatsworth, CA, USA) according to the manufacturer's instructions.

#### Example 2

This Example describes the immunization of mice. Mice are susceptible to infection by RSV as described in ref. 16.

For intramuscular (i.m) immunization, the anterior tibialis anterior muscles of groups of 9 BALB/c mice (male, 6-8 week old) (Jackson Lab., Bar Harbor, ME, USA) were bilaterally injected with 2 x 50  $\mu$ g (1  $\mu$ g/ $\mu$ L in

PBS) of pXL1-4, respectively. Five days prior to DNA injection, the muscles were treated with 2 x 50  $\mu$ L (10  $\mu$ M in PBS) of cardiotoxin (Latoxan, France). Pretreatment of the muscles with cardiotoxin has been reported to increase DNA uptake and to enhance the subsequent immune responses by the intramuscular route (ref. 24). These animals were similarly boosted a month later. Mice in the control group were immunized with a placebo plasmid containing identical vector backbone sequences without the RSV F gene according to the same schedule. For intradermal (i.d.) immunization, 100  $\mu$ g of pXL2 (2  $\mu$ g/ $\mu$ L in PBS) were injected into the skin 1-2 cm distal from the tail base. The animals were similarly boosted a month later.

Seventy-five days after the second immunization, mice were challenged intranasally with  $10^{5.4}$  plaque forming units (pfu) of mouse-adapted RSV, A2 subtype (obtained from Dr. P. Wyde, Baylor College of Medicine, Houston, TE, USA). Lungs were aseptically removed 4 days later, weighed and homogenized in 2 mL of complete culture medium. The number of pfu in lung homogenates was determined in duplicates as previously described (ref. 19) using vaccine quality Vero cells. These data were subjected to statistic analysis using SigmaStat (Jandel Scientific Software, Guelph, Ont. Canada).

Sera obtained from immunized mice were analyzed for anti-RSV F antibody titres (IgG, IgG1 and IgG2a, respectively) by enzyme-linked immunosorbent assay (ELISA) and for RSV-specific plaque-reduction titres. ELISA were performed using 96-well plates coated with immunoaffinity purified RSV F protein (50 ng/mL) and 2-fold serial dilutions of immune sera. A goat anti-mouse IgG antibody conjugated to alkaline phosphatase (Jackson ImmunoRes., Mississauga, Ont., Canada) was used as secondary antibody. For the measurement of IgG1 and

IgG2a antibody titres, the secondary antibodies used were monospecific sheep anti-mouse IgG1 (Serotec, Toronto, Ont., Canada) and rat anti-mouse IgG2a (Zymed, San Francisco, CA, USA) antibodies conjugated to alkaline phosphatase, respectively. Plaque reduction titres were determined according to Prince et al (ref. 19) using vaccine quality Vero cells. Four-fold serial dilutions of immune sera were incubated with 50 pfu of RSV, Long strain (ATCC) in culture medium at 37°C for 1 hr in the presence of 5% CO<sub>2</sub>. Vero cells were then infected with the mixture. Plaques were fixed with 80% methanol and developed 5 days later using a mouse anti-RSV-F monoclonal IgG1 antibody and donkey antimouse IgG antibody conjugated to peroxidase (Jackson ImmunoRes., Mississauga, Ont. Canada). The RSV-specific plaque reduction titre was defined as the dilution of serum sample yielding 60% reduction in the number of plaques.

Both ELISA and plaque reduction assays were performed in duplicates and data are expressed as the means of two determinations. These data were subjected to statistic analysis using SigmaStat (Jandel Scientific Software, Guelph, Ont. Canada).

To examine the induction of RSV-specific CTL following DNA immunization, spleens from 2 immunized mice were removed to prepare single cell suspensions which were pooled. Splenocytes were incubated at  $2.5 \times 10^6$  cells/mL in complete RPMI medium containing 10 U/mL murine interleukin 2 (IL-2) with  $\gamma$ -irradiated (3,000 rads) syngeneic splenocytes ( $2.5 \times 10^6$  cells/mL) infected with 1 TCID<sub>50</sub>/cell RSV (Long strain) for 2 hr. The source of murine IL-2 was supernatant of a mouse cell line constitutively secreting a high level of IL-2 obtained from Dr. H. Karasuyama of Basel Institute for Immunology (ref. 20). CTL activity was tested 5 days following the *in vitro* re-stimulation in a standard 4 hr chromium release assay. Target cells were 5 <sup>51</sup>Cr-

labelled uninfected BALB/c fibroblasts (BC cells) and persistently RSV-infected BCH14 fibroblasts, respectively. Washed responder cells were incubated with  $2 \times 10^3$  target cells at varying effector to target ratios in 200  $\mu$ L in 96-well V-bottomed tissue-culture plates for 4 hr at 37°C. Spontaneous and total chromium releases were determined by incubating target cells with either medium or 2.5% Triton-X 100 in the absence of responder lymphocytes. Percentage specific chromium release was calculated as  $(\text{counts-spontaneous counts})/(\text{total counts-spontaneous counts}) \times 100$ . Tests were performed in triplicates and data are expressed as the means of three determinations. For antibody blocking studies in CTL assays, the effector cells were incubated for 1 hr with 10  $\mu$ g/ml final of purified mAb to CD4 (GK1.5) (ref. 21) or mAb against murine CD8 (53-6.7) (ref. 22) before adding chromium labelled BC or BCH4 cells. To determine the effect of anti-class I MHC antibodies on CTL killing, the chromium labelled target cells BC or BCH4 were incubated with 20  $\mu$ L of culture supernate of hybridoma that secretes a mAb that recognizes K<sup>d</sup> and D<sup>d</sup> of class I MHC (34-1-2S) (ref. 23) prior to the addition of effector cells.

### Example 3

25 This Example describes the immunogenicity and protection by polynucleotide immunization by the intramuscular route.

To characterize the antibody responses following i.m. DNA administration, immune sera were analyzed for anti-RSV F IgG antibody titre by ELISA and for RSV-specific plaque reduction titre, respectively. All four plasmid constructs were found to be immunogenic. Sera obtained from mice immunized with pXL1-4 demonstrated significant anti-RSV F IgG titres and RSV-specific plaque reduction titres as compared to the placebo group

(Table 1 below) ( $P < 0.0061$  and  $< 0.0001$ , respectively, Mann-Whitney Test). However, there is no significant difference in either anti-RSV F IgG titre or RSV-specific plaque reduction titre among mice immunized 5 with either pXL1, pXL2, pXL3 or pXL4.

To evaluate the protective ability of pXL1-4 against primary RSV infection of the lower respiratory tract, immunized mice were challenged intranasally with mouse-adapted RSV and viral lung titres post challenge 10 were assessed. All four plasmid constructs were found to protect animals against RSV infection. A significant reduction in the viral lung titre was observed in mice immunized with pXL1-4 as compared to the placebo group ( $P < 0.0001$ , Mann-Whitney Test). However, varying degrees 15 of protection were observed depending on the plasmid. In particular, PXL1 was more protective than pXL3 ( $P = 0.00109$ , Mann-Whitney Test), and pXL4 more than pXL3 ( $P = 0.00125$ ), whereas only pXL2 induced complete protection. This conclusion was confirmed by another 20 analysis with number of fully protected mice as end point (Fisher Exact Test). Constructs pXL1, pXL2 or pXL4 conferred a higher degree of protection than pXL3 ( $P < 0.004$ , Fisher Exact Test) which was not more effective than placebo. Only pXL2 conferred full 25 protection in all immunized mice.

The above statistical analysis revealed that PXL1 conferred more significant protection than pXL3. The former expresses the truncated and secretory form and the latter the intact membrane anchored form of the RSV 30 F protein. Furthermore, pXL4 was shown to be more protective than pXL3. The difference between these two constructs is the presence of the intron II sequence in pXL4. Construct pXL2 which expresses the secretory form of the RSV-F in the context of the intron II sequence 35 was the only plasmid that confers complete protection in all immunized mice.

Example 4

This Example describes the influence of the route of administration of pXL2 on its immunogenicity and protective ability.

5       The i.m. and i.d. routes of DNA administration were compared for immunogenicity in terms of anti-RSV F antibody titres and RSV-specific plaque reduction titres. Analyses of the immune sera (Table 2 below) revealed that the i.d. route of DNA administration was  
 10 as immunogenic as the i.m. route as judged by anti-RSV F IgG and IgG1 antibody responses as well as RSV-specific plaque reduction titres. However, only the i.m. route induced significant anti-RSV F IgG2a antibody responses, whereas the IgG2a isotype titre was negligible when the  
 15 i.d. route was used. The i.m. and i.d. routes were also compared with respect to the induction of RSV-specific CTL. Significant RSV-specific CTL activity was detected in mice immunized intramuscularly. In contrast, the cellular response was significantly lower in mice  
 20 inoculated intradermally (Table 3 below). In spite of these differences, protection against primary RSV infection of the lower respiratory tract was observed in both groups of mice immunized via either route (Table 4 below). The CTL induced by RSV-F DNA are classical CD8+  
 25 class I restricted CTL. The target cells, BCH4 fibroblasts express class I MHC only and do not express class II MHC. Further, prior incubation of BCH4 target cells with anti class-I MHC antibodies significantly blocked the lytic activity of RSV-F DNA induced CTL  
 30 line. While anti-CD8 antibody could partially block lysis of BCH4 cells, antibody to CD4 molecule had no effect at all (Table 5 below). Lack of total blocking by mAb to CD8 could either be due to CTL being CD8 independent (meaning that even though they are CD8+ CTL,  
 35 their TCR has enough affinity for class I MHC+peptide and it does not require CD8 interaction with the alpha 3

of class I MHC) or the amount of antibody used in these experiments was limiting. There was no detectable lysis of YAC-1 (NK sensitive target) cells (data not shown).

Example 5

5        This Example describes immunization studies in cotton rats using pXL2.

         The immune response of cotton rats to DNA immunization was analyzed by the protocol shown in Table 6 below. On day -5, 40 cotton rats were randomly  
10 selected and divided into 8 groups of 5. Cotton rats in groups 1 and 7 were inoculated intramuscularly (i.m.) into the tiberlia arteria (TA) muscles bilaterally with cardiotoxin (1.0  $\mu$ M). On day -1, the cotton rats in group 8 were inoculated in the TA muscles with  
15 bupivacaine (0.25%). On day 0, several animals in each group were bled to determine levels of RSV-specific antibodies in the serum of the test animals prior to administration of vaccines. All of the animals were then inoculated i.m. or intradermally (i.d.) with 200  $\mu$ g  
20 of plasmid DNA, placebo (non-RSV-specific DNA), 100 median cotton rat infectious doses (CRID50; positive control) of RSV, or of formalin inactivated RSV prepared in Hep-2 tissue culture cells and adjuvanted in alum. Forty-four days later the cotton rats in groups 1 & 7  
25 were reinoculated with cardiotoxin in the TA muscles. Four days later (48 days after priming with vaccine), the animals in group 8 were reinoculated with bupivacains in the TA muscle of the right leg. The next day, (seven weeks after priming with vaccine) all of the  
30 animals were bled and all, except those in the group given live RSV, were boosted with the same material and doses used on day 0. 29 days later, each cotton rat was bled and then challenged intranasally (i.n.) with 100 CRID50 RSV A2 grown in Hep-2 tissue culture cells. Four  
35 days after this virus challenge (day +88) all of the cotton rats were killed and their lungs removed. One



lobe from each set of lungs was fixed in formalin and then processed for histologic evaluation of pulmonary histopathology. The remaining lobes of lung will be assessed for the presence and levels of RSV. Each of  
5 the sera collected on days 0, 49 and 78 were tested for RSV-neutralizing activity, anti-RSV fusion activity and RSV-specific ELISA antibody.

The RSV neutralizing titres on day +49 and +78 are shown in Tables 7(a) below and 7(b) below respectively.  
10 As can be seen from the results shown in Table 7(a), on day +49 the animals immunized with live RSV and DNA immunization had substantial RSV serum neutralizing titres. The animals immunized with formalin-inactivated RSV had a neutralizing titre equivalent to the placebo  
15 group on day +49 but following boosting titres by day +78 had reached 5.8 ( $\log_{10}/0.05$ ). Boosting had no significant effect upon animals immunized with live RSV or by i.m. plasmid immunization.

RSV titres in nasal washes (upper respiratory  
20 tract) on day +82 are shown in Table 8 below. RSV titres in the lungs (lower respiratory tract) on day +82 are shown in Table 9 below. All of the vaccines provided protection against lung infection but under these conditions, only live virus provided total  
25 protection against upper respiratory tract infection.

The lungs from the cotton rats were examined histologically for pulmonary histopathology and the results are shown in Table 10 below. With the exception of lung sections obtained from Group 9 which were  
30 essentially free of inflammatory cells or evidence of inflammation, and those from Group 3, which exhibited the maximal pulmonary pathology seen in this study, all of the sections of lung obtained from the other groups looked familiar, i.e. scattered inflammatory cells were  
35 present in most fields, and there was some thickening of septae. These are evidence of mild inflammatory

diseases. Large numbers of inflammatory cells and other evidence of inflammation were present in sections of lung from Group 3 (in which formalin-inactivated [FI] RSV vaccine was given prior to virus challenge). This result indicated that immunization with plasmid DNA expressing the RSV F protein does not result in pulmonary histopathology different from the placebo, whereas FI-RSV caused more severe pathology.

#### Example 6

10 This Example describes the determination of local lung cytokine expression profile in mice immunized with pXL2 after RSV challenge.

Balb/C mice were immunized at 0 and 6 weeks with 100 µg of pXL2, prepared as described in Example 1, and  
15 challenged with RSV i.n. at 10 weeks. Control animals were immunized with FI-RSV and live RSV and challenged with RSV according to the same protocol. Four days post viral challenge, lungs were removed from immunized mice and immediately frozen in liquid nitrogen. Total RNA  
20 was prepared from lungs homogenized in TRIzol/β-mercaptoethanol by chloroform extraction and isopropanol precipitation. Reverse transcriptase-polymerase chain reaction (RT-PCR) was then carried out on the RNA samples using either IL-4, IL-5 or IFN-γ specific  
25 primers from Clone Tech. The amplified products were then liquid-hybridized to cytokine-specific <sup>32</sup>P-labeled probes from Clone Tech, resolved on 5% polyacrylamide gels and quantitated by scanning of the radioactive signals in the gels. Three mouse lungs were removed  
30 from each treatment group and analyzed for lung cytokine expression for a minimum of two times. The data is presented in Figure 9 and represents the means and standard deviations of these determinations.

As may be seen from the data presented in Figure 9:

1. Immunization with live RSV intranasally (i.n.) resulted in a balanced cytokine profile (IFN- $\gamma$ , IL-4 and IL-5), whereas that with FI-RSV intramuscularly (i.m.) resulted in a Th2 predominance (elevated IL-4 and IL-5). These results are similar to what were reported in the literature.
2. Immunization with pXL2 containing the secretory (sec.) form of FI via either the i.m. or intradermal (i.d.) route gave rise to a balanced cytokine profile similar to that with live RSV immunization.
3. The magnitude of the cytokine responses with i.m. and i.d. immunization using pXL2 expressing a secretory form of the protein is significantly higher than that with live RSV immunization.

#### Example 7

This Example describes the construction of a plasmid vector encoding the RSV F protein and containing the 5' UTR and signal peptide of Herpes Simplex Virus I (HSV I)gD.

Plasmid p82M35B was prepared following the scheme shown in Figure 10. Plasmid pVR1012 (Vical) (Figure 11; SEQ ID No: 6) containing the CMV promoter, intron A, and the BGH poly A sequences, was linearized with restriction enzyme Pst I and made blunt ended with T4 DNA polymerase. The rabbit  $\beta$ -globin intron II sequence was retrieved from plasmid pSG5 (Stratagene; ref. 14) by Cla I and Eco RI digestion, and the 0.6 kb fragment was isolated and made blunt ended by treatment with Klenow fragment polymerase. The rabbit  $\beta$ -globin intron II fragment was then ligated to the Pst I/blunt ended VR1012 plasmid (Fig. 10). This vector was then restricted with Eco RV and dephosphorylated.

The secreted form of RSV F was isolated from plasmid pXL2 (Example 1; Fig. 5) by digestion with Sal I, made blunt end by treatment with Klenow fragment polymerase, then restricted with Kpn I to produce a 5' Kpn I, 3' blunt ended fragment. The HSV gD sequence was synthesized as a synthetic oligonucleotide having the DNA (SEQ ID No: 7) and derived amino acid (SEQ ID No: 8) sequence shown in Figure 12.

The gD oligonucleotide has a 5' blunt end and 3' Kpn I recognition sequence. A three-way ligation was performed with the isolated RSV F fragment, gD oligo and the VR1012 plasmid, to produce plasmid p82M35B (Fig. 10).

#### Example 8

This Example illustrates the expression and secretion of RSV F protein *in vitro*.

BHK cells were transfected with either p82M35B, its counterpart containing the autologous RSV F signal peptide (pXL2) or the vector backbone alone (placebo) using Lipofectin (Gibco/BRL). Forty-eight hours post transfection, supernatant fractions were recovered and subjected to RSV F protein quantification using a F-specific enzyme-linked immunoabsorbent assay (ELISA). Three independent transfection assays were performed for each vector.

ELISAs were performed using one affinity-purified mouse monoclonal anti-RSV F antibody (2 µg/ml) as the capturing reagent and another biotinolated monoclonal anti-RSV F antibody (0.1 µg/ml) as the detection reagent. Horseradish peroxidase-labelled avidin (Pierce) was subsequently used. The RSV F standard protein used was purified from detergent-lysates of cultured virus by immunoaffinity chromatography.

Table 11 (below) shows the results obtained. As seen in Table II, compared to placebo, both p82M35B and pXL2 mediated significant F protein expression/secretion

from the BHK cells 48 hours post transfection. Furthermore, a markedly higher level of the F protein was consistently detected in the supernatant fraction of p82M35B-transfected BHK cells than that of pXL2-transfected cells, representing a 5.4-fold improvement over the latter. These results indicate that replacement of the coding sequence for the autologous RSV F signal peptide with that for the 5'UTR and signal peptide of HSV I gD significantly enhanced F protein expression/secretion in vitro.

#### Example 9

This Example illustrates immunogenicity studies carried out using p82M35B.

Tibialis anterior muscles of BALB/c mice (male, 6 to 8 weeks old) (Jackson Lab., Bar Harbor, ME, USA) were bilaterally injected with 2 x 50 µg (1 µg/µL in PBS) of p82M35B, pXL2 or the vector backbone alone (placebo). In some groups, 5 days prior to DNA injection, the muscles were treated with 2 x 50 µL (10 µM in PBS) of cardiotoxin (Latoxan, France) to increase DNA uptake and enhance immune responses as reported by Davis et al., (ref. 24). The animals were boosted with the same dose of plasmid DNA 6 weeks later. Mice in the positive control group were immunized intranasally (i.n.) with 10<sup>6</sup> plaque forming units (pfu) of a clinical RSV strain of the A2 subtype grown in Hep2 cells kindly provided by Dr. B. Graham (ref. 16).

Antisera obtained from immunized mice were analyzed for anti-RSV F IgG antibody titres using specific ELISA and for RSV-specific plaque-reduction titres. ELISAs were performed using 96-well plates coated with immunoaffinity-purified RSV F protein (50 ng/mL) and 2-fold serial dilutions of immune sera. A goat anti-mouse IgG antibody conjugated to alkaline phosphatase (Jackson ImmunoRes., Mississauga, Ont., Canada) was used as secondary antibody. Plaque reduction titres were

determined according to Prince et al. (ref. 19) using vaccine-quality Vero cells. Four-fold serial dilutions of immune sera were incubated with 50 pfu of the RSV Long strain (ATCC) in culture medium at 37°C for 1 hr in the presence of 5% CO<sub>2</sub> and the mixtures were used to infect Vero cells. Plaques were fixed with 80% methanol and developed 5 days later using a mouse anti-RSV F monoclonal IgG1 antibody and donkey anti-mouse IgG antibody conjugated to peroxidase (Jackson ImmunoRes. Mississauga, Ont.). The RSV-specific plaque reduction titre was defined as the dilution of serum sample yielding 60% reduction in plaque number. Both ELISAs and plaque reduction assays were performed in duplicate and data are expressed as the means of two determinations.

The results of these studies are set forth in Table 12 below. For the induction of serum antibody responses (Table 12), p82M35B is effective without the need of cardiotoxin pretreatment under the DNA dose and immunization regimen used, resulting in anti-F IgG titre of  $7.2 \pm 1.1$  ( $\log_2$  titre/100) and RSV-specific plaque reduction titre of  $11.8 \pm 0.9$  ( $\log_2$ ) after two immunizations. In contrast, the antibody titres elicited by pXL2 in the absence of the cardiotoxin pretreatment were significantly lower (IgG titre of  $2.9 \pm 2.3$  and plaque reduction titre of  $8.2 \pm 1.9$ ). However, serum antibody responses elicited by pXL2 were significantly improved with the cardiotoxin pretreatment step (IgG titre of  $7.4 \pm 1.1$  and plaque reduction titre of  $10.5 \pm 0.8$ ). The placebo was unable to elicit a detectable serum antibody response in the absence or presence of the cardiotoxin pretreatment step.

This trend was extendible to results of the protection study (Table 12). Vector p82M35B conferred full protection against RSV infection of lungs in the absence of the cardiotoxin pretreatment. In contrast,

pXL2 only conferred partial protection under the same conditions. However, full protection was achieved with the pXL2 vector when cardiotoxin pretreatment step was included in the immunization regimen. No protection was  
5 observed with the placebo with or without the cardiotoxin pretreatment step.

These results show the replacement of the coding sequence for the autologous RSV F signal peptide with that for the 5'UTR and signal peptide of HSV I gD  
10 resulted in significant enhancement in not only F protein expression/secretion assessed in vitro (Example 8), but also immunogenicity to the F protein as well as protective ability against RSV infection assessed in the mouse model.

15

#### SUMMARY OF THE DISCLOSURE

In summary of this disclosure, the present invention provides certain novel vectors containing genes encoding an RSV F proteins, methods of immunization using such vectors and methods of diagnosis  
20 using such vectors. Modifications are possible within the scope of this invention.

Table 1: Immunogenic and Protective Abilities of pXL1-4 Mice via the i.m. Route

Plasmid DNA Immunogen	No. Mice	Mean Anti-RSV F ELISA Titre(IgG)* (Log <sub>2</sub> /100±SD)	Mean Plaque Reduction Titre* (Log <sub>4</sub> ±SD)	Post RSV Challenge	
				Mean Virus Lung Titre# (pfu/g lung (Log <sub>10</sub> ±SD)	No. Fully Protected Mice**
pXL1	8	3.00±1.85	3.74±0.98	0.72±0.99	5
pXL2	9	5.78±1.72	4.82±0.51	0.00±0.00	9
pXL3	8	3.75±2.05	4.59±1.16	2.77±0.72	0
pXL4	9	5.44±1.13	5.18±0.43	0.66±1.00	6
Placebo**	12	0.58±2.89	0.18±0.62	3.92±0.27	0

\* These sets of data from sera obtained 1 week prior to the viral challenge

# Detection sensitivity of the assay was 10<sup>1.96</sup> pfu/g lung.

\*\* The term, fully protected mice, refers to animals with no detectable RSV in lungs post challenge.



Table 2. Immunogenicity of pXL2 in Mice\*

Route	No. Mice	Mean Anti-RSV F ELISA Titre (Log <sub>2</sub> /100 + SD)			Mean Plaque Reduction Titre (Log <sub>4</sub> ± SD)
		IgG	IgG1	IgG2a	
i.m	8	7.63 ± 0.92	4.25 ± 1.91	4.38 ± 1.92	4.18 ± 0.88
i.d.	7	7.00 ± 1.00	5.00 ± 1.00	0.14 ± 0.38	3.65 ± 0.59
Placebo(i.m.)	9	0.50 ± 0.51	0.00 ± 0.00	0.00 ± 0.00	0.18 ± 0.50

\* These sets of data are from sera obtained 1 week prior to the viral challenge.

Table 3. Induction of RSV-specific CTL Following DNA Immunization\*

Route	E:T Ratio	% Specific Lysis	
		BC	BCH4
i.m.	200:1	23.3	100.6
	100:1	17.0	62.4
	50:1	19.9	64.1
	25:1	22.3	46.4
i.d.	100:1	20.9	26.1
	50:1	21.7	19.1
	25:1	7.1	7.0
	12.5:1	2.8	2.3

\* These set of data were obtained from immunized mice immediately prior to RSV challenge.

Table 4. Immunoprotective Ability of pXL2 in Mice

Route	No. Mice	Post RSV Challenge	
		Mean Virus Lung Titre* (pfu/g lung)	No. Fully Protected Mice#
i.m.	8	0.00 ± 0.00	8
i.d.	7	0.43 ± 1.13	6
Placebo (i.m.)	9	4.30 ± 0.22	0

\* Detection sensitivity of the assay was  $10^{1.69}$  pfu/g lung.

# The term, fully protected mice, refers to animals with no detectable RSV in lungs post challenge.

Table 5. RSV specific CTL included by i.m. DNA immunization are class I restricted CTL

E:T Ratio	BCH4	BCH4 + anti-CD4	BCH4 + anti-CD8	BCH4 + anti-class I MHC
100:1	52.03	54.3	39.4	8.6
50:1	44.4	47.2	27.4	6.2
25:1	28.6	26.3	14.8	1
12.5:1	18.2	15	8	-2.7

Table 6

Group	Antigen	RSV-specific dose	Inoc. route	Pretreatment/Adjuvant	Day 0	Day 49	Day 78	Day 88
1	Placebo	0	I.M.	Cardiotoxin	Prebleed, several cotton rats per group; prime all animals	Bleed all animals; boost all except those in group 2	Challenge with RSV A2 I.N. after bleeding all	Harv. animals and do histologic evaluation, pulmonary virus titers, antibodies
2	Live RSV	100 CRID50	I.N.	None				
3	FI-RSV		I.M.	Alum				
5	pXL2	200 µg	I.M.	None				
6	pXL2	200 µg	I.D.	None				
7	pXL2	200 µg	I.M.	Cardiotoxin				
8	pXL2	200 µg	I.M.	Bupivacaine				

Table 7(a). RSV Serum Neutralizing Titers on Day 49

Group	Antigen	RSV-specific dose	Inoc. route	Nt. antibody titer ( $\log_2/0.05$ ml) in CR no.				Mean titer $\log_2/0.05$	Stand. Dev.
				1	2	3	4		
1	Placebo	0	I.M.	4	3	2	2	2.75	1.0
2	Live RSV	100 CRID50	I.N.	9	9	9	9	9	0.0
3	FI-RSV		I.M.	0	4	2	2	2.0	1.6
5	pXL2	200 $\mu$ g	I.M.	9	8	8	7	8.0	0.8
6	pXL2	200 $\mu$ g	I.D.	5	2	5	5	4.3	1.5
7	pXL2	200 $\mu$ g	I.M.	8	8	9	9	8.5	0.6
8	pXL2	200 $\mu$ g	I.M.	8	9	6	6	7.3	1.5

Table 7(b). RSV Serum Neutralizing Titers on Day 78

Group	Antigen	RSV-specific dose	Inoc. route	Nt. antibody titer ( $\log_2/0.05$ ml) in CR no.				Mean titer $\log_2/0.05$	Stand. Dev.
				1	2	3	4		
1	Placebo	0	I.M.	3	2	4	Died	3.0	1.0
2	Live RSV	100 CRID50	I.N.	8	9	8	9	8.5	0.6
3	FI-RSV		I.M.	8	4	6	5	5.8	1.7
5	pXL2	200 $\mu$ g	I.M.	7	8	8	8	7.8	0.5
6	pXL2	200 $\mu$ g	I.D.	8	6	6	Died	6.7	1.2
7	pXL2	200 $\mu$ g	I.M.	8	9	9	8	8.7	0.6
8	pXL2	200 $\mu$ g	I.M.	8	7	9	9	8.3	1.0

Table 8. RSV Titers in Nasal Washes on Day 82

Group	Antigen	RSV-specific dose	Inoc. route	RSV titer ( $\log_{10}/0.05$ ml) in cotton rat no.				Mean titer $\log_{10}/0.05$	Stand. Dev.
				1	2	3	4		
1	Placbo	0	I.M.	3.4	3.3	3.3	Died	3.3	0.1
2	Live RSV	100 CRID50	I.N.	0	0	0	0	0.0	0.0
3	FI-RSV		I.M.	0	0	2.8	0	0.7	1.4
5	pXL2	200 $\mu$ g	I.M.	3.3	2.3	3.3	2.3	2.8	0.6
6	pXL2	200 $\mu$ g	I.D.	N.D.	N.D.	N.D.	Died	N.D.	N.D.
7	pXL2	200 $\mu$ g	I.M.	2.3	0	0	3.2	1.4	1.6
8	pXL2	200 $\mu$ g	I.M.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

N.D. = non-determined

Table 9. Titers in Lungs on Day 82

Group	Antigen	RSV-specific dose	Inoc. route	RSV titer ( $\log_{10}/g$ lung) in cotton rat no.				Mean titer $\log_{10}/0.05$	Stand. Dev.
				1	2	3	4		
1	Placebo	0	I.M.	4.7	4.2	3.7	Died	4.2	0.5
2	Live RSV	100 CRID50	I.N.	0	0	0	0	0.0	0.0
3	FL-RSV	$10^6$ PFU	I.M.	0	0	0	0	0.0	0.0
5	pXL2	200 $\mu g$	I.M.	0	2.2	0	0	0.6	1.1
6	pXL2	200 $\mu g$	I.D.	0	2.2	2.7	3.2	2.0	N.D.
7	pXL2	200 $\mu g$	I.M.	0	0	0	0	0.0	0.0
8	pXL2	200 $\mu g$	I.M.	0	0	0	0	0.0	N.D.

N.D. = non-determined



Table 10. Summary of Histopathology Results Seen in Sections of Cotton Rat Lung.

Group	Treatment	Major Observations & Comments
1.	Placebo + RSV	Scattered individual and groups of macrophages and polymorphonuclear neutrophils (PMN) in all fields. Overt thickening of septae. Occasional pyknotic cells seen. Overall: mild to moderate inflammation.
2.	Live RSV	Isolated macrophages seen in most fields. Scattered PMN. Overall: minimal inflammation
3.	FI-RSV + RSV	Virtually every field contains numerous mononuclear cells & PMN. Pyknotic cells and debris common. Thickened septae. Evidence of exacerbated disease.
5.	Plasmid + RSV	Isolated macrophages seen in most fields. Occasional PMN seen. Very similar to live virus group.
6.	Plasmid i.d. + RSV	Isolated macrophages seen in most fields. Occasional PMN seen.
7.	Plasmid + CT + RSV	Isolated mononuclear cells and PMN seen in most fields.
8.	Plasmid + Biv + RSV	Scattered mononuclear cells and PMN seen in most fields.
9.	Normal CR Lung	Few leukocytes evidence. Airy, open appearance. Thin septae.

CT = carditoxin

Biv = bupivacaine

Table 11. Expression/Secretion of the RSV F protein from BHK cells  
(48 hr post transfection)

Plasmid Construct	F Protein Secretion (mean $\pm$ S.D.) (ng/mL)	Magnitude of Improvement
Placebo	0.0 $\pm$ 0.0	
p82M35B	32.1 $\pm$ 2.06	5.4 x (over pXL2)
pXL2	5.9 $\pm$ 0.6	

Table 12. Immunoprotective Ability of DNA-F in BALB/c Mice

Immunogen	Anti-F IgG Titre Log <sub>2</sub> (titre/100) 10 weeks	RSV-Specific Plaque Reduction Titre (Log <sub>2</sub> titre)	Mean Virus Lung Titre* (pfu/g lung) (Log <sub>2</sub> 10 ± SD)	No. Fully protected # No. Immunized
Placebo (i.m.)	0.0 ± 0.0	0.0 ± 0.0	4.3 ± 0.5	0/6
p82M35B (i.m.)	7.2 ± 1.1	11.8 ± 0.9	0.0 ± 0.0	6/6
pXL2 (i.m.)	2.9 ± 2.3	8.2 ± 1.9	2.9 ± 1.7	1/6
pXL2 + cardiotoxin	7.4 ± 1.1	10.5 ± 0.8	0.0 ± 0.0	6/6
RSV (i.n.)	8.5 ± 2.7	12.4 ± 0.7	0.0 ± 0.0	6/6

\* Sensitivity of assay: 10<sup>1.69</sup> pfu/g lung.

# The term, fully protected mice, refers to animals with no detectable RSV in the lungs 4 days post viral challenge.

REFERENCES

1. McIntosh K., Canock, R.M. In: Fields BN, Knipe, DM, editors. Virology. New York: Raven Press: 1990: 1045-1072.
2. Katz SL., In: New Vaccine Development establishing priorities. Vol. 1. Washington: National Academic Press: 1985: 397-409.
3. Wertz GW, Sullender WM., Biotechnology 1992; 20: 151-176.
4. Johnson et al., J. Virol 1987, 61: 3163-3166.
5. Pemberton et al., J. Gen Virol. 1987, 68: 2177-2182.
6. Crowe, J.E., Vaccine 1995, 13: 415-421.
7. WO 90/11092
8. WO 94/21797
9. Ulmer, Current Opinion, Invest Drugs, 1993, 2: 983-989.
10. Tang et al., Nature 1992, 356: 152-154.
11. Furth et al. Analytical Biochemistry, 1992, 205: 365-368.
12. Pizzorno et al., J. Virol. 1988, 62: 1167-1179.
13. Chapman, B.S.; Thayer, R.M.; Vincent, K.A. and Haigwood, N.L., Nucl. Acids. Res. 1991, 19: 3979-3986.
14. Green, S. Isseman, I., and Sheer, E., Nucl. Acids. Res. 1988, 16: 369.
15. Breathnach, R. and Harris, B.A., Nucl. Acids Res. 1983, 11: 7119-7136.
16. Graham, B.S.; Perkins M.D.; Wright, P.F. and Karzon, D.T. J. Mod. Virol. 1988 26: 153-162.
17. Nabel, G.J. 1993, Proc. Natl. Acad. Sci. USA 90: 11307-11311.
18. Du, R.P et al. 1994., Biotechnology 12: 813-818.
19. Prince, G.A. et al, 1978. Ame. J. Pathol. 93: 771-790.

20. Karasuyama & Melchers, Eur. J. Immunol. 18, 97-104, 1988.
21. Wilde, David et al. 1983. J. Immunol. 131: 2178-2183.
22. Ledbetter, J.A., Rouse R., Micklem, H. 1980, J. Exp. Med. 152: 280-295
23. Ozato Kerko et al. 1982, Transplantation 34: 113-118.
24. Davis et al., Vaccine 1994, 12: 1503-1509

CLAIMS

What we claim is:

1. An immunogenic composition for in vivo administration to a host for the generation in the host of a protective immune response to RSV F protein, comprising a non-replicating vector comprising:

a first nucleotide sequence encoding an RSV F protein or a RSV F protein fragment that generates antibodies and/or cytotoxic T-lymphocytes (CTLs) that specifically react with RSV F protein;

a promoter sequence operatively coupled to the first nucleotide sequence for expression of said RSV F protein in the host, and

a second nucleotide sequence located between said first nucleotide sequence and said promoter sequence to enhance the immunoprotective ability of said RSV F protein when expressed in vivo from said vector in a host; and

a pharmaceutically-acceptable carrier therefor.

2. The composition of claim 1 wherein said first nucleotide sequence encodes a full-length RSV F protein.

3. The composition of claim 1 wherein said first nucleotide sequence encodes a RSV F protein from which the transmembrane region is absent.

4. The composition of claim 1 wherein said first nucleotide sequence encodes a RSV F protein fragment lacking a transmembrane coding region.

5. The composition of claim 1 wherein said promoter sequence is an immediate early cytomegalovirus promoter.

6. The composition of claim 1 wherein said second nucleotide sequence comprises a pair of splice sites to prevent aberrant mRNA splicing, whereby substantially all RNA transcribed from the vector encodes an RSV F protein.

7. The composition of claim 6 wherein said second nucleotide sequence is located between said first nucleotide sequence and said promoter sequence.

8. The composition of claim 7 wherein said second nucleotide sequence is that of rabbit  $\beta$ -globin intron II.

9. The composition of claim 1 wherein said first nucleotide sequence encodes a RSV F protein fragment lacking an autologous RSV F signal peptide sequence and includes a sequence encoding a heterologous signal peptide which enhances the level of expression of RSV F protein.

10. The composition of claim 9 wherein said first nucleotide sequence encoding a signal peptide encodes HSV I gD.

11. The composition of claim 10 wherein said first nucleotide sequence encodes a RSV F protein fragment lacking a transmembrane coding region.

12. The composition of claim 1 wherein said non-replicating vector is plasmid pXL2 as shown in Figure 5.

13. The composition of claim 1 wherein said non-replicating vector is plasmid pXL4 as shown in Figure 7.

14. The composition of claim 1 wherein said non-replicating vector is plasmid p82M35B as shown in Figure 10.

15. A method of immunizing a host against disease caused by infection with respiratory syncytial virus (RSV), which comprises administering to said host an effective amount of a non-replicating vector comprising a first nucleotide sequence encoding an RSV F protein or a RSV F protein fragment that generates antibodies and/or cytotoxic T-lymphocytes (CTLs) that specifically react with RSV F protein and a promoter sequence operatively coupled to said first nucleotide sequence for expression of said RSV F protein in said host.

16. The method of claim 15, wherein the first nucleotide sequence encodes a full-length RSV F protein.

17. The method of claim 15 wherein said first nucleotide sequence encodes a RSV F protein fragment lacking a transmembrane region.

18. The method of claim 15 wherein the first nucleotide sequence encodes a RSV F protein fragment lacking an autologous RSV F signal peptide sequence and includes a sequence encoding a heterologous signal peptide which enhances the level of expression of RSV F protein.

19. The composition of claim 18 wherein the first nucleotide sequence encodes a signal peptide encodes HSV I gD.

20. The composition of claim 19 wherein the first nucleotide sequence encodes a RSV F protein fragment lacking a transmembrane coding region.

21. The method of claim 15 wherein said host is a human.

22. The method of claim 21 wherein said promoter sequence is an immediate early cytomegalovirus promoter.

23. The method of claim 15 wherein said non-replicating vector further comprises a second nucleotide sequence located adjacent said first nucleotide sequence to enhance the immunoprotective ability of said RSV F protein when expressed *in vivo* from said vector in said host.

24. The method of claim 23 wherein said promoter sequence is an immediate early cytomegalovirus promoter.

25. The method of claim 24 wherein said second nucleotide sequence comprises a pair of splice sites to prevent aberrant mRNA splicing, whereby substantially all mRNA transcribed from the vector encodes an RSV F protein.

26. The method of claim 25 wherein said second nucleotide sequence is located between said first nucleotide sequence and said promoter sequence.



27. The method of claim 26 wherein said second nucleotide sequence is that of rabbit  $\beta$ -globulin intron II.

28. The method of claim 23 wherein said vector is pXL2 as shown in Figure 5.

29. The method of claim 23 wherein said vector is pXL4 as shown in Figure 7.

30. The method of claim 23 wherein said vector is p82M35B as shown in Figure 10.

31. A method of using a gene encoding an RSV F protein or a RSV protein fragment that generates antibodies and/or cytotoxic T-lymphocytes (CTLs) that specifically react with RSV F protein to produce an immune response in a host, which comprises:

isolating said gene;

operatively linking said gene to at least one control sequence to produce a non-replicating vector, said control sequence directing expression of said RSV F protein when said vector is introduced into a host to produce an immune response to said RSV F protein or fragment thereof; and

introducing said vector into the host.

32. The method of claim 31 wherein said gene encoding an RSV F protein encodes an RSV F protein lacking the transmembrane region.

33. The method of claim 32 wherein said at least one control sequence comprises the immediate early cytomegalovirus promoter.

34. The method of claims 31 wherein said gene coding an RSV F protion encodes a RSV F protein fragment lacking an autologous RSV F signal peptide sequence and includes a sequence encoding a heterologous signal peptide which enhance the level of expression of RSV F protein.

35. The method of claims 34 wherein said gene coding an RSV F protion encoding a signal peptide encodes HSV I gD.

36. The method of claim 35 wherein said gene encoding a RSV F protein encodes a RSV F protein fragment lacking a transmembrane coding region.

37. The method of claim 36 wherein said at least one control sequence comprises the immediately early cytomegalovirus promoter.

38. The method of claim 37 including the step of:

operatively linking said gene to an immunoprotective enhancing sequence to produce an enhanced immunoprotection to said RSV F protein in said host.

39. The method of claim 38 wherein said immunoprotective enhancing sequence is introduced into said vector between said control sequence and said gene.

40. The method of claim 39 wherein said immunoprotection enhancing sequence comprises a pair of splice sites to prevent aberrant mRNA splicing whereby substantially cell transcribed mRNA encodes an RSV F protein.

41. The method of claim 40 wherein said immunoprotection enhancing sequence is that of rabbit  $\beta$ -globin intron II.

42. The method of claim 31 wherein said gene is contained within a plasmid vector selected from the group consisting of pXL2, pXL4 and p82M35B.

43. A method of producing a vaccine for protection of a host against disease caused by infection with respiratory syncytial virus (RSV), which comprises:

isolating a first nucleotide sequence encoding an RSV F protein or a RSV F protein fragment that generates antibodies and/or cytotoxic T-lymphocytes (CTLs) that specifically react with RSV F protein;

operatively linking said first nucleotide sequence to at least one control sequence to produce a non-replicating vector, the control sequence directing expression of said RSV F protein when introduced into a

host to produce an immune response to said RSV F protein;

operatively linking said first nucleotide sequence to a second nucleotide sequence to enhance the immunoprotective ability of said RSV F protein when expressed *in vivo* from the vector in a host, and

formulating said vector as a vaccine for *in vivo* administration.

44. The method of claim 43 wherein said non-replicating vector is a plasmid vector selected from the group consisting of pXL2, pXL4 and p82M35B.

45. A vaccine produced by the method of claim 43.

46. A method of producing a vaccine for protection of a host against disease caused by infection with respiratory syncytial virus (RSV), which comprises:

isolating a first nucleotide sequence encoding an RSV F protein from which the transmembrane region is absent;

operatively linking said first nucleotide sequence to at least one control sequence to produce a non-replicating vector, the control sequence directing expression of said RSV F protein when introduced into a host to produce an immune response to said RSV F protein; and

formulating said vector as a vaccine for *in vivo* administration.

47. The method of claim 46 wherein said vector is a plasmid vector selected from group consisting of pXL2 and p82M35B.

48. A vaccine produced by the method of claim 46.

49. A method of determining the presence of a respiratory syncytial virus (RSV) F protein in a sample, comprising the steps of:

(a) immunizing a host with a non-replicating vector comprising a first nucleotide sequence encoding an RSV F protein or a RSV F protein

fragment that generates antibodies that specifically react with RSV F protein and a promoter sequence operatively coupled to said first nucleotide sequence for expression of said RSV F protein in said host to produce antibodies specific for the RSV F protein;

(b) isolating the RSV F protein specific antibodies;

(c) contacting the sample with the isolated antibodies to produce complexes comprising any RSV F protein present in the sample and said isolated RSV F protein-specific antibodies; and

(d) determining production of the complexes.

50. The method of claim 49 wherein said vector is a plasmid vector selected from the group consisting of pXL1, pXL2, pXL3, pXL4 and p82M35B.

51. A diagnostic kit for detecting the presence of an RSV F protein in a sample, comprising:

(a) a non-replicating vector comprising a first nucleotide sequence encoding an RSV F protein or a RSV F protein fragment that generates antibodies that specifically react with RSV F protein and a promoter sequence operatively coupled to said first nucleotide sequence for expression of said RSV F protein in a host immunized therewith to produce antibodies specific for the RSV F protein;

(b) isolation means to isolate said RSV F protein-specific antibodies;

(c) contacting means to contact the isolated RSV F specific antibodies with the sample to produce a complex comprising any RSV F protein present in the sample and RSV F protein specific antibodies, and

(d) identifying to determine production of the complex.

52. The diagnostic kit of claim 51 wherein said vector is a plasmid vector selected from the group consisting of pXL1, pXL2, pXL3, pXL4 and p82M35B.

53. A method for producing antibodies specific for an F protein of RSV comprising:

(a) immunizing a host with an effective amount of a non-replicating vector comprising a first nucleotide sequence encoding an RSV F protein lacking a transmembrane region and a promoter sequence operatively coupled to said first nucleotide sequence for expression of said RSV F protein in said host to produce antibodies specific for the F protein; and

(b) isolating the antibodies from the host.

54. A method of producing monoclonal antibodies specific for an F protein of RSV comprising the steps of:

(a) constructing a non-replicating vector comprising a first nucleotide sequence encoding an RSV F protein and a promoter sequence operatively coupled to said first nucleotide sequence for expression of said RSV F protein; and, optionally,

a second nucleotide sequence located adjacent said first nucleotide sequence to enhance the immunoprotective ability of said RSV F protein when expressed *in vivo* from said vector in a host.

(b) administering the vector to at least one mouse to produce at least one immunized mouse;

(c) removing B-lymphocytes from the at least one immunized mouse;

(d) fusing the B-lymphocytes from the at least one immunized mouse with myeloma cells, thereby producing hybridomas;

(e) cloning the hybridomas;

(f) selecting clones which produce anti-F protein antibody;

(g) culturing the anti-F protein antibody-producing clones; and

(h) isolating anti-F protein monoclonal antibodies from the cultures.

ABSTRACT OF THE DISCLOSURE

Non-replicating vectors containing a nucleotide sequence coding for an F protein of respiratory syncytial virus (RSV) and a promoter for such sequence, preferably a cytomegalovirus promoter, are described for *in vivo* immunization. The nucleotide sequence encoding the RSV F protein may lack a sequence encoding the homologous signal peptide but possessing a heterologous signal peptide enhancing RSV F protein expression. Such non-replicating vectors, including plasmids, also may contain a further nucleotide sequence located adjacent to the RSV F protein encoding sequence to enhance the immunoprotective ability of the RSV F protein when expressed *in vivo*. Such non-replicating vectors may be used to immunize a host against disease caused by infection with RSV, including a human host, by administration thereto, and may be formulated as immunogenic compositions with pharmaceutically-acceptable carriers for such purpose. Such vectors also may be used to produce antibodies for detection of RSV infection in a sample.

Docket No.  
1038-922 MIS:jb

## Declaration and Power of Attorney For Patent Application

### English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

**NUCLEIC ACID RESPIRATORY SYNCYTIAL VIRUS VACCINES**

the specification of which

(check one)

☒ is attached hereto.

☐ was filed on \_\_\_\_\_ as United States Application No. or PCT International  
Application Number \_\_\_\_\_  
and was amended on \_\_\_\_\_

(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Priority Not Claimed

(Number)

(Country)

(Day/Month/Year Filed)

☐

(Number)

(Country)

(Day/Month/Year Filed)

☐

(Number)

(Country)

(Day/Month/Year Filed)

☐



I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

\_\_\_\_\_  
(Application Serial No.)

\_\_\_\_\_  
(Filing Date)

\_\_\_\_\_  
(Application Serial No.)

\_\_\_\_\_  
(Filing Date)

\_\_\_\_\_  
(Application Serial No.)

\_\_\_\_\_  
(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

**08/896,500**

**July-18-1997**

**Pending**

\_\_\_\_\_  
(Application Serial No.)

\_\_\_\_\_  
(Filing Date)

\_\_\_\_\_  
(Status)  
(patented, pending, abandoned)

**08/659,939**

**June-7-1996**

**Patented**

\_\_\_\_\_  
(Application Serial No.)

\_\_\_\_\_  
(Filing Date)

\_\_\_\_\_  
(Status)  
(patented, pending, abandoned)

**08/476,397**

**June 7, 1995**

**Pending**

\_\_\_\_\_  
(Application Serial No.)

\_\_\_\_\_  
(Filing Date)

\_\_\_\_\_  
(Status)  
(patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

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Fifth inventor's signature	Date
Residence	
Citizenship	
Post Office Address	

Full name of sixth inventor, if any	
Sixth inventor's signature	Date
Residence	
Citizenship	
Post Office Address	

RESTRICTION MAP OF THE RSV F GENE

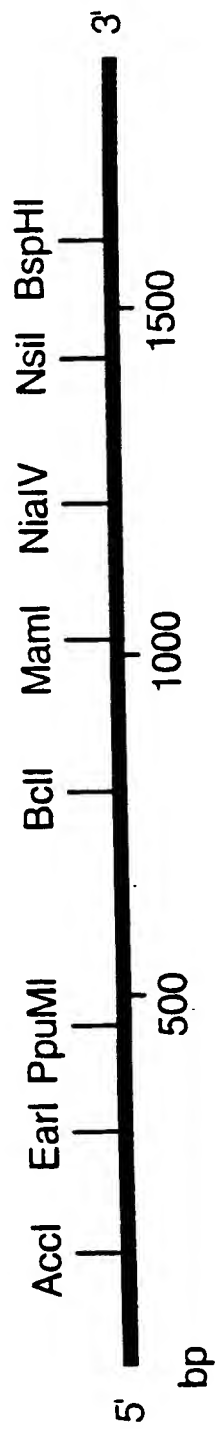


FIG.1

FIG. 2A

NUCLEOTIDE SEQUENCE OF THE RSV F GENE.

5' MET GLU LEU PRO ILE LEU LYS ALA ASN ALA ILE THR THR ILE LEU ALA VAL THR PHE  
 ATGGAGTTGCCAATCCTCAAGCAAATGCAATTACCAACAATCCCTCGCTGCAGTCACATTT  
 TACCTCAACGGTTAGGAGTTTCGTTTACGTTAATGGTGTAGGAGCGACGTCAGTGTA  
 10 20 30 40 50 60

CYS PHE ALA SER SER GLN ASN ILE THR GLU GLU PHE TYR GLN SER THR CYS SER ALA VAL  
 TGCTTTGCTTCTAGTCAAAACATCACTGAAGAAATTTATCAATCAACATGCAGTGCAGTT  
 ACGAAACGAAGATCAGTTTGTAGTGACTTCTTAAATAAGTTAGTTGTACGTCACGTCAA  
 70 80 90 100 110 120

SER LYS GLY TYR LEU SER ALA LEU ARG THR GLY TRP TYR THR SER VAL ILE THR ILE GLU  
 AGCAAAGGCTATCTTAGTGCTCTAAGAACTGGTGGTATACCTAGTGTATATACTATAGAA  
 TCGTTTCCGATAGAAATCAGGAGATTCTTGACCAACCATATGATCACAAATATGATATCTT  
 130 140 150 160 170 180

LEU SER ASN ILE LYS GLU ASN LYS CYS ASN GLY THR ASP ALA LYS VAL LYS LEU MET LYS  
 TTAAGTAATATCAAGGAAAATAAGTGTAATGGAACAGATGCTAAGGTAAATTTGATGAAA  
 AATTCAATTATAGTTCCCTTTTATTCACATTACCTTGCTACGATTCCCATTTTAACTACTTT  
 190 200 210 220 230 240

GLN GLU LEU ASP LYS TYR LYS ASN ALA VAL THR GLU LEU GLN LEU MET GLN SER THR  
 CAAGAAATTAGATAAATATAAAAATGCTGTAAACAGAAATTCAGTTGCTCATGCAAGCACA  
 GTTCTTAATCTATTATATTTTACGACATTGTCTTAAACGTCAACGAGTACGTTTCGTGT  
 250 260 270 280 290 300

PRO ALA ALA ASN ASN ARG ALA ARG ARG GLU LEU PRO ARG PHE MET ASN TYR THR LEU ASN  
 CCAGCAGCAACAATCGAGCCAGAGAGAACTACCAAGGTTTATGAATTATACACTCAAC  
 GGTCGTCGTTTGTAGCTCGGTCCTTCTCTTGATGGTTCCAAATACTTAATATGTGAGTTG  
 310 320 330 340 350 360

FIG. 2B

F2-F1CLEAVAGE SITE

ASN THR LYS LYS THR ASN VAL THR LEU SER LYS LYS ARG LYS ARG ARG↓PHE LEU GLY PHE  
 AATACCAGGTTTGGTTACATTGTAACATTAAGCAAGAAAGGAAA8AAGATTTCCTTGGTTT  
 TTATGGTTT 370 380 390 400 410 420

LEU LEU GLY VAL GLY SER ALA ILE ALA SER GLY ILE ALA VAL SER LYS VAL LEU HIS LEU  
 TTGTAGGTGTTGGATCTGCAATCGCCAGTGGCATTTGCTGTATCTAAGTCCCTGCACCTTA  
 AACAAATCCACAAACCTAGACGTTAGCGGTACCCGTAACGACATAGATTCCAGGACGTGAAT  
 430 440 450 460 470 480

GLU GLY GLU VAL ASN LYS ILE LYS SER ALA LEU LEU SER THR ASN LYS ALA VAL VAL SER  
 GAAGGAGAAGTGAACAAGATCAAAAGTCTCTACTATCCACAACAAGCCGTAGTCAGC  
 CTTCCCTCTTCACCTTGTCTAGTTTTCACGAGATGATAGGTGTTTGTTCGGCATCAGTCG  
 490 500 510 520 530 540

LEU SER ASN GLY VAL SER VAL LEU THR SER LYS VAL LEU ASP LEU LYS ASN TYR ILE ASP  
 TTATCAAAATGGAGTTAGTGTCTTAACCAGCAAAAGTGTAGACCTCAAAAACCTATATAGAT  
 AATAGTTTACCTCAATCACAGAAATTGGTCGTTTCACAAATCTGGAGTTTGTGATATATCTA  
 550 560 570 580 590 600

LYS GLN LEU LEU PRO ILE VAL ASN LYS GLN SER CYS ARG ILE SER ASN ILE GLU THR VAL  
 AAACAATTGTTACCTATTGTGAATAAGCAAGCTGCAGAAATATCAAAATAGAAACTGTG  
 TTTGTAAACAATGGATAACACTTATTCTGTTTCGACGTCTTATAGTTTATATCTTTGACAC  
 610 620 630 640 650 660

ILE GLU PHE GLN HIS LYS ASN ASN ARG LEU LEU GLU ILE THR ARG GLU PHE SER VAL ASN  
 ATAGAGTCCACAACAAGAAACAACAGACTACTAGAGATTACCAAGGAATTTAGTGTAAAT  
 TATCTCAAGGTGTTTCTTGTGTTCTGATGATCTCTAATGGTCCCTTAAATCACAATTA  
 670 680 690 700 710 720

ALA GLY VAL THR THR PRO VAL SER THR TYR MET LEU THR ASN SER GLU LEU LEU SER LEU  
 GCAGGTGTAACCTACACCTGTAAGCAGCTTACATGTTAACTAATAAGTGAATTTATGTCATTA  
 CGTCCACATTGATGTGGACATTTCGTGAATGTACAATTGATTATCATTAAATAACAGTAAT  
 730 740 750 760 770 780

FIG. 2C

ILE ASN ASP MET PRO ILE THR ASN ASP GLN LYS LYS LEU MET SER ASN ASN VAL GLN ILE  
 ATCAATGATATGCCCTATAACAAATGATCAGAAAAAGTTAATGTCCAAACAATGTTCAAATA  
 TAGTTACTATACGGGATATTGTTTACTAGTCTTTTTCAAATTACAGGTTGTTACAAGTTTAT  
 790 800 810 820 830 840  
 VAL ARG GLN GLN SER TYR SER ILE MET SER ILE ILE LYS GLU GLU VAL LEU ALA TYR VAL  
 GTTAGACAGCAAGTTACTCTATCATGTCCATAATAAAAGAGGAAGTCTTAGCATATGTA  
 CAATCTGTCTGTTTCAATGAGATAGTACAGGTATTATTCTCTCCCTTCAGAAATCGTATACAT  
 850 860 870 880 890 900  
 VAL GLN LEU PRO LEU TYR GLY VAL ILE ASP THR PRO CYS TRP LYS LEU HIS THR SER PRO  
 GTACAAATTACCACATATATGGTGTGATAGATACACCTTGTGTGGAATTACACACATCCCCCT  
 CATGTTAATGCTGATATACCACACTATCTATGTGGAACAACCTTTAATGTGTAGGGGA  
 910 920 930 940 950 960  
 LEU CYS THR THR ASN THR LYS GLU GLY SER ASN ILE CYS LEU THR ARG THR ASP ARG GLY  
 CTATGTACAACCAACACAAAGAGGGTCAACACATCTGTTTAAACAAGAACTGACAGAGGA  
 GATACATGTTGGTGTGTTTCTTCCAGTTTGTAGACAAAATGTTCTTGACTGTCTCCT  
 970 980 990 1000 1010 1020  
 TRP TYR CYS ASP ASN ALA GLY SER VAL SER PHE PHE PRO GLN ALA GLU THR CYS LYS VAL  
 TGGTACTGTGACAAATGCAGGATCAGTATCTTCTTCCCAAGCTGAACACATGTAAAGTT  
 ACCATGACACTGTTACGTCTCTAGTCATAGAAAGAGGGTGTTCGACTTTGTACATTTTCAA  
 1030 1040 1050 1060 1070 1080  
 GLN SER ASN ARG VAL PHE CYS ASP THR MET ASN SER LEU, THR LEU PRO SER GLU VAL ASN  
 CAATCGAATCGAGTATTTTGTGACACAAATGAACAGTTTAAACATTTACCAAGTGAAGTAAAT  
 GTTAGCTTAGCTCATAAACACACTGTGTACTTTGTCAAAATTTGTAATGGTTCACTTTCATTTA  
 1090 1100 1110 1120 1130 1140  
 LEU CYS ASN VAL ASP ILE PHE ASN PRO LYS TYR ASP CYS LYS ILE MET THR SER LYS THR  
 CTCTGCAATGTTGACATATTTCAATCCCAAAATATGATTGTAAAAATATGACTTCAAAAAACA  
 GAGACGTTACAACTGTATAAAGTTAGGGTTTATATACTAACATTTTAATACCTGAAGTTTGTG  
 1150 1160 1170 1180 1190 1200

ASP VAL SER SER VAL ILE THR SER LEU GLY ALA ILE VAL SER CYS TYR GLY LYS THR  
GATGTAAGCAGCTCCGTTATCATCTCTCTAGGAGCCATTGTGTCTATGCTATGCGCAAACT  
CTACATTCGTCGAGGCAATAGTGAGAGATCCCTCGGTAACACACAGTACGATACCGTTTGA  
1210 1220 1230 1240 1250 1260

LYS CYS THR ALA SER ASN LYS ASN ARG GLY ILE ILE LYS THR PHE SER ASN GLY CYS ASP  
AAATGTACAGCATCCCAATAAAATCCGTGGAAATCATAAAGACATTTTCTAAACGGGTGTGAT  
TTTACATGTCTCGTAGGTTATTTTTAGCACCTTAGTATTTCTGTAAAAGATTGCCCCACACTA  
1270 1280 1290 1300 1310 1320

TYR VAL SER ASN LYS GLY **VAL** ASP THR VAL SER VAL GLY ASN THR LEU TYR TYR VAL ASN  
TATGTATCAAAATAAAGGGGTGGACACTGTGTCTGTAGGTAAACACATTTATATTATGTAAAT  
ATACATAGTTTATTTCCCCACCTGTGACACAGACATCCCATTTGTGTAATAATAACATTTA  
1330 1340 1350 1360 1370 1380

LYS GLN GLU GLY LYS SER LEU TYR VAL LYS GLY GLU PRO ILE ASN PHE TYR ASP PRO  
AAGCAAGAAGGCAAAAGTCTCTCTATGTAAAGGTGAACCAATAATAATTTCTATGACCCCA  
TTCGTTCTTCCGTTTTCAGAGATACATTTTCCACTTGGTTATTTAAAGATACTGGGT  
1390 1400 1410 1420 1430 1440

LEU VAL PHE PRO SER ASP GLU PHE ASP ALA SER ILE SER GLN VAL ASN GLU LYS ILE ASN  
TTAGTATTTCCCTCTGTGATGAATTTTGATGCAATCAATATCTCAAGTCAATGAGAAAGATTAAAC  
AATCATAAAGGGAGACTACTTAACCTACGTAAGTATAGAGTTTCAGTTACTCTTCTAATTG  
1450 1460 1470 1480 1490 1500

GLN SER LEU ALA PHE ILE ARG LYS SER ASP GLU LEU LEU HIS ASN VAL ASN ALA GLY LYS  
CAGAGTTTAGCATTATTCGTAAATCCGATGAATTTATTTACATAATGTAAATGCTGGTAA  
GTCTCAAAATCGTAAATAAGCATTTAGGCTACTTAATAATGTTATTAACAATTCGACCATTT  
1510 1520 1530 1540 1550 1560

SER THR THR ASN ILE MET ILE THR THR ILE ILE GLU ILE ILE VAL ILE LEU SER  
TCAACCACAAATATCATGATGATACTACTATAATTTATAGAGATTATAGTAAATTTGTTATCA  
AGT TGGTGTTTATAGTACTATTGATGATTAATATCTCTAATATCATTAATAACAATAGT  
1570 1580 1590 1600 1610 1620

FIG. 2D



LEU ILE ALA VAL GLY LEU LEU TYR CYS LYS ALA ARG SER THR PRO VAL THR LEU SER  
 TTAATTGCTGTTGGACTGCTCCTATACTGTAAGGCCAGAACACACCACTCACTAAGC  
 AATTAAACGACAACCTGACGAGGATATGACATTCGGGTCTTCGTCGTCAGTGTGATTTCG  
 1630 1640 1650 1660 1670 1680

LYS, ASP GLN LEU SER GLY ILE ASN ASN ILE ALA PHE SER ASN  
 AAGGATCAACTGAGTGAGTGGTATAAATAAATATGCAATTTAGTAACCTGAATAAATAAGCACCT  
 TTCCTAGTTGACTCACCATAATTTATTAATAACGTAAATCATTCGACTTATTTTATCCTGCGA  
 1690 1700 1710 1720 1730 1740

AATCATGTTCTTACAAATGGTTTACTATCTGCTCATAGACAACCCATCTATCATTTGGATT  
 TTAGTACAAGAAATGTTACCAAAATGATAGACGAGTATCTGTTGGGTAGATAGTAACCTAAA  
 1750 1760 1770 1780 1790 1800

TCTTAAATCTGAACCTTCATCGAAACTCTTATCTATAAACCATCTCACTTACACTATTTA  
 AGAATTTTAGACTTGAAGTAGCTTTTGAGAAATAGATATTTTGGTAGAGTGAATGTGATAAAT  
 1810 1820 1830 1840 1850

AGTAGATTCCCTAGTTTATAGTTATAT 3'  
 TCATCTAAGGATCAAAATATCAATATA  
 1870 1880

NUCLEOTIDE SEQUENCE OF THE RSV F GENE. THE cDNA SEQUENCE IS SHOWN IN THE PLUS (mRNA)  
 STRAND SENSE IN THE 5' TO 3' DIRECTION. THE SIGNAL PEPTIDE (SP) AND THE TRANSMEMBRANE (TM)  
 ANCHOR DOMAIN ARE UNDERLINED. THE PREDICTED F2-F1 CLEAVAGE SITE IS INDICATED BY THE ARROW  
 (↓).

FIG. 2E

FIG. 3A

NUCLEOTIDE SEQUENCE OF THE RSV F GENE.

5' MET GLU LEU PRO ILE LEU LYS ALA ASN ALA ILE THR THR ILE LEU ALA VAL THR PHE  
 ATGGAGTTGCCAATCCTCAAAGCAAATGCAATFACCACAATCCTCGCTGCAGTCACATTT  
 TACCTCAACGGTTAGGAGTTTTCGTTTACGTTAATGGTGTAGGAGCGACGTCAGTGTA  
 10 20 30 40 50 60

CYS PHE ALA SER SER GLN ASN ILE THR GLU GLU PHE TYR GLN SER THR CYS SER ALA VAL  
 TGCTTTGCTTCTAGTCAAAACATCACCCTGAAGAATTTTATCAATCAACATGCAGTGCAGTT  
 ACGAAACGAAGATCAGTTTGTAGTGACTTCTTAAATAATAGTTAGTTGTACGTCACGTC  
 70 80 90 100 110 120

SER LYS GLY TYR LEU SER ALA LEU ARG THR GLY TRP TYR THR SER VAL ILE THR ILE GLU  
 AGCAAAGGCTATCTTAGTGCTCTAAGAAGTGTGGTACTAGTGTATATACTAATATAGAA  
 TCGTTTCCGATAGAAATCACCAGATTCTTGACCAACCATATGATCACAATATATGATATCTT  
 130 140 150 160 170 180

LEU SER ASN ILE LYS GLU ASN LYS CYS ASN GLY THR ASP ALA LYS VAL LYS LEU MET LYS  
 TTAAGTAATATCAAGGAAATAAGTGTAAATGGAACAGATGCTAAGGTAAATGTATGATAA  
 AATTCAATTATAGTTCCCTTTTATTCACATTACCTTGCTACGATTCCATTTTAACTACTTT  
 190 200 210 220 230 240

GLN GLU LEU ASP LYS TYR LYS ASN ALA VAL THR GLU LEU GLN LEU MET GLN SER THR  
 CAAGAATTAGATAAATATAAAATGCTGTAAACAGAAATTCAGATTGCTCATGCAAGCACA  
 GTTCTTAATCTATTATATTTTACGACATTGCTCTTAACGTCACGAGTACGTTTCGTGT  
 250 260 270 280 290 300

PRO ALA ALA ASN ASN ARG ALA ARG ARG GLU LEU PRO ARG PHE MET ASN TYR THR LEU ASN  
 CCAGCAGCAACAATCGAGCCAGAAGAACTACCAAGGTTTATGAATTATACACTCAAC  
 GGTCGTCGTTTGTAGCTCGGCTTCTCTTGTATGTTCCAAATACTTAATATGTGAGTTG  
 310 320 330 340 350 360

F2-F1CLEAVAGE SITE

ASN THR LYS LYS THR ASN VAL THR LEU SER LYS LYS ARG LYS ARG ARG PHE LEU GLY PHE  
AATACCAAAAACCAATGTAACATTAAAGCAAGAAAAGGAAA8AAGATTCTTGGTGTATTA  
TTATGGTTTCTTGGTTACATTGTAAATTCGTTCTTTTCCCTTTCTTCTTCAAAGAACCAAAA  
370 380 390 400 410 420

LEU LEU GLY VAL GLY SER ALA ILE ALA SER GLY ILE ALA VAL SER LYS VAL LEU HIS LEU  
TTGTAGGTGTTGGATCTGCAATCGCCAGTGGCATTGCTGTATCTAAGGTCCTGCACCTTA  
AACAAATCCACAACCTAGACGTTAGCGGTACCGTAACGACATAGATTCCAGGACGTGAAT  
430 440 450 460 470 480

GLU GLY GLU VAL ASN LYS ILE LYS SER ALA LEU LEU SER THR ASN LYS ALA VAL SER  
GAAGGAGAAAGTGAACAAGATCAAAAGTGCTCTACTATCCACAACAAGCCGTAGTCAGC  
CTTCCCTCTTCACCTTGTTCTAGTTTTCACGAGATGATAGGTGTTGTTCCGGCATCAGTCG  
490 500 510 520 530 540

LEU SER ASN GLY VAL SER VAL LEU THR SER LYS VAL LEU ASP LEU LYS ASN TYR ILE ASP  
TTATCAAAATGGAGTTAGTGCTTAAACCAGCAAAAGTGTAGACCTCAAAAACCTATATAGAT  
AATAGTTTACCTCAATCACAGAAATTGGTCTGTTTCACAAATCTGGAGTTTGTGATATATCTTA  
550 560 570 580 590 600

LYS GLN LEU LEU PRO ILE VAL ASN LYS GLN SER CYS ARG ILE SER ASN ILE GLU THR VAL  
AAACAATTTACCTATTGTGAATAAGCAAGCTGCAGAAATATCAAAATATAGAAAACCTGTG  
TTTGTAAACAATGGATAACACTTATTCGTTTCGACGCTTATAGTTTATATATCTTTTGACAC  
610 620 630 640 650 660

ILE GLU PHE GLN HIS LYS ASN ASN ARG LEU LEU GLU ILE THR ARG GLU PHE SER VAL ASN  
ATAGAGTTCCAACAACAAGAACACAGACTACTAGAGATTACCAGGAAATTTAGTGTTAAT  
TATCTCAAGGTGTTTCTTGTCTGTGATCTCTAATGGTCCCTTAAATCACAATTA  
670 680 690 700 710 720

ALA GLY VAL THR THR PRO VAL SER THR TYR MET LEU THR ASN SER GLU LEU LEU SER LEU  
GCAGGTGTAACACCTGTAAAGCACTTACATGTTAACTAATAGTGAATTAATGTCATTA  
CGTCCACATTGATGTGGACATTCTGTGAATGTACAATTGATTATCACTTAAATAACAGTAAAT  
730 740 750 760 770 780

FIG. 3C

ILE ASN ASP MET PRO ILE THR ASN ASP GLN LYS LYS LEU MET SER ASN ASN VAL GLN ILE  
 ATCAATGATATGCCCTATAACAATAATGATCAGAAAAAGTTAATGTCCAAACAATGTTCAAATA  
 TAGTACTATACGGATATTTGTTTACTAGTCTTTTTCAAATTACAGGTTGTTACAAGTTTAT 840  
 790 800 810 820 830  
 VAL ARG GLN GLN SER TYR SER ILE MET SER ILE LYS GLU GLU VAL LEU ALA TYR VAL  
 GTTAGACAGCAAAAGTTACTCTATCATGTCCATAATAAAAGAGGAAAGTCTTTAGCATATGTA  
 CAATCTGTCGTTTCAATGAGATAGTACAGGTATTTTCTCCTTTCAGAAATCGTATACAT 900  
 850 860 870 880 890  
 VAL GLN LEU PRO LEU TYR GLY VAL ILE ASP THR PRO CYS TRP LYS LEU HIS THR SER PRO  
 GTACAATTAACCACTATATGGTGTGATAGATACACCTTGTGGAATTAACACACATCCCCCT  
 CATGTTAATGGTGATATACCACTATCTATGTGGAACAACCTTTAATGTGTAGGGGA 960  
 910 920 930 940 950  
 LEU CYS THR THR ASN THR LYS GLU GLY SER ASN ILE CYS LEU THR ARG THR ASP ARG GLY  
 CTATGTACAACCAACACAAAGAGGGTCAACATCTGTTTAACAAGAACTGACAGAGGA  
 GATACATGTTGGTGTGTTTCTTCTCCAGTTGTAGACAAAATGTTCTTGACTGTCTCCT 1020  
 970 980 990 1000 1010  
 TRP TYR CYS ASP ASN ALA GLY SER VAL SER PHE PHE PRO GLN ALA GLU THR CYS LYS VAL  
 TGGTACTGTGACAAATGCAGGATCAGTATCTTTCTTCCCACAAGCTGAAACATGTAAAGTT  
 ACCATGACACTGTTACGTCCTAGTCATAGAAAGAGGGTGTTCGACTTTGTACATTTCAA 1080  
 1030 1040 1050 1060 1070  
 GLN SER ASN ARG VAL PHE CYS ASP THR MET ASN SER LEU THR LEU PRO SER GLU VAL ASN  
 CAATCGAATCGAGTATTTGTGACACAAATGAACAGTTTAACATTACCAGTGAAGTAAAT  
 GTTAGCTTAGCTCATAAACACTGTGTACTTGTCAAAATGTAAATGGTTCACCTTCATTTA 1140  
 1090 1100 1110 1120 1130  
 LEU CYS ASN VAL ASP ILE PHE ASN PRO LYS TYR ASP CYS LYS ILE MET THR SER LYS THR  
 CTCTGCAATGTTGACATATTCAAATCCCAATATGATGTGTAATAATGACTTCAAAAAACA  
 GAGACGTTACAACACTGTATAAGTTAGGGTTTATACTAACAATTTTAATACTGAAGTTTTCG 1200  
 1150 1160 1170 1180 1190

FIG. 3D

ASP VAL SER SER VAL ILE THR SER LEU GLY ALA ILE VAL SER CYS TYR GLY LYS THR  
GATGTAAGCAGCTCCGTATACACATCTCTAGAGCCCATTTGTGTCATGCTATGGCAAAACT  
CTACATTCGTCGAGGCAATAGTGTAGAGATCCTCGGTAACACACAGTACGATACCGTTTGA  
1210 1220 1230 1240 1250 1260

LYS CYS THR ALA SER ASN LYS ASN ARG GLY ILE ILE LYS THR PHE SER ASN GLY CYS ASP  
AAATGTACAGCATCCAATAAATAATCGTGAATCATATAAGACATTTTCTAAACGGGTGTGAT  
TTTACATGTCGTAGGTTATTTTTCAGCACCTTAGTATTTCTGTAAAGATTTGCCACACTA  
1270 1280 1290 1300 1310 1320

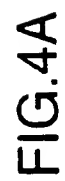
TYR VAL SER ASN LYS GLY VAL ASP THR VAL SER VAL GLY ASN THR TYR TYR VAL ASN  
TATGTATCAATAAAGGGGTGGACACTGTGCTGTAGGTAACACATTTATATATGTAAAT  
ATACATAGTTTATTTCCCCACCCTGTGACACAGACATCCATTGTGTAATAATAACATTTA  
1330 1340 1350 1360 1370 1380

LYS GLN GLU GLY LYS SER LEU TYR VAL LYS GLY GLU PRO ILE ILE ASN PHE TYR ASP PRO  
AAGCAAGAAGGCAAAAGTCTCTATGTAAAGGTGAACCAATAAJAAATTTCTATGACCCCA  
TTCGTTCTTCCCGTTTTCAGAGATACATTTTCCCACTTGGTTATTTAAAGATAC'TGGGT  
1390 1400 1410 1420 1430 1440

LEU VAL PHE PRO SER ASP GLU PHE ASP ALA SER ILE SER GLN VAL ASN GLU LYS ILE ASN  
TTAGTATTTCCCTCTGATGAATTTGATGCATCAATATCTCAAGTCAATGAGAAGATTAAC  
AATCATAGGGGAGACTACTTAAACTACGTAGTTATAGAGTTCAGTTACTCTTCTAATTG  
1450 1460 1470 1480 1490 1500

GLN SER LEU ALA PHE ILE ARG LYS SER ASP GLU LEU LEU HIS ASN VAL ASN ALA GLY LYS  
CAGAGTTTAGCATTTATTTGTAATCCGATGAATTTATACATAATGTAATGCTGGTAAA  
GTCTCAAATCGTAAATAAGCATTTAGGCTACTTAATAATGTATTACATTTACGACCATTT  
1510 1520 1530 1540 1550 1560

SER THR THR ASN ILE MET Thr Stop Stop Bam HI  
TCAACCACAAATATCATGACTTGATAATGAGGATCC  
AGTTGGTGTATTATAGTACTGAACATATTACTCTCTAGG  
1570



**FIG. 4A**

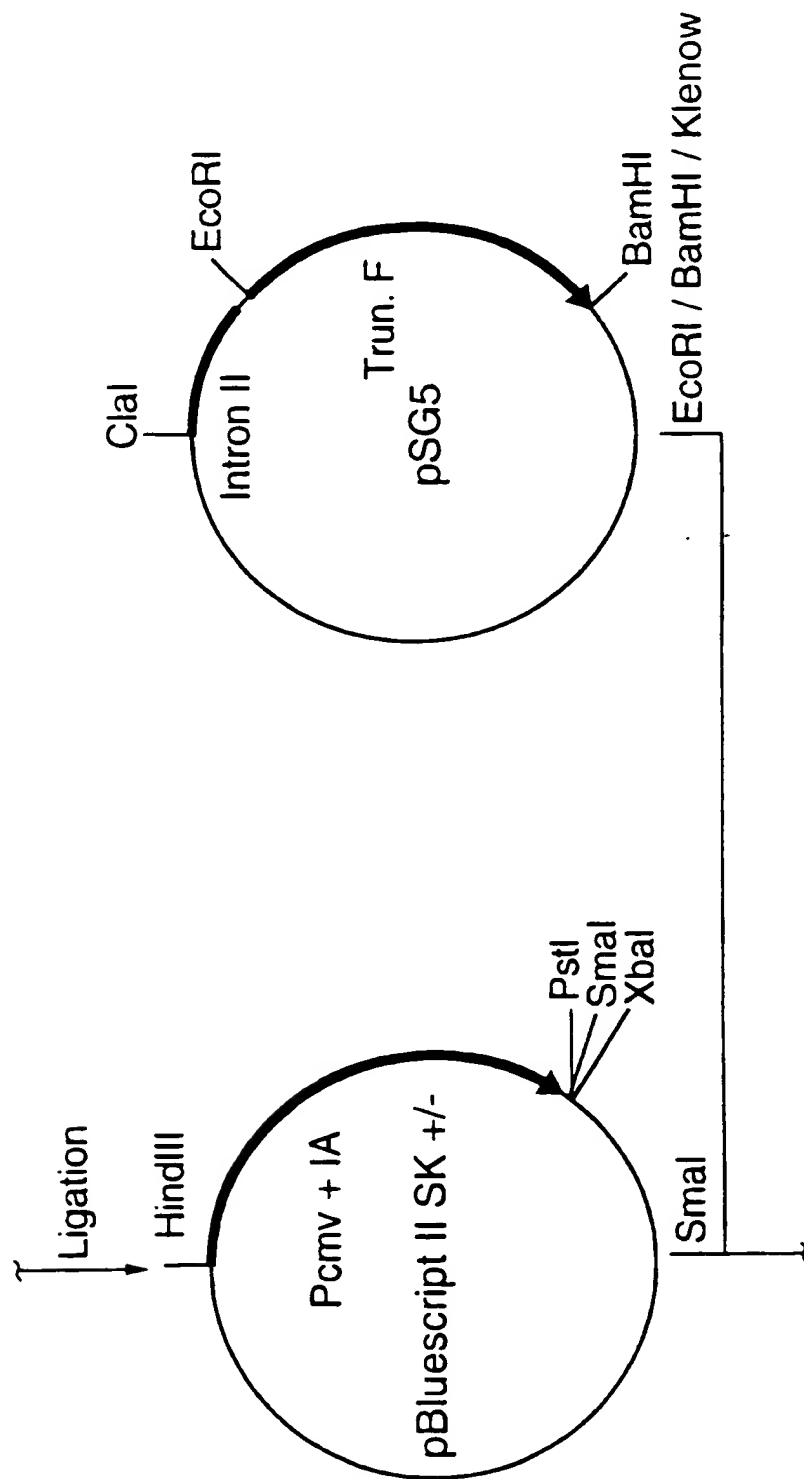


FIG.4B

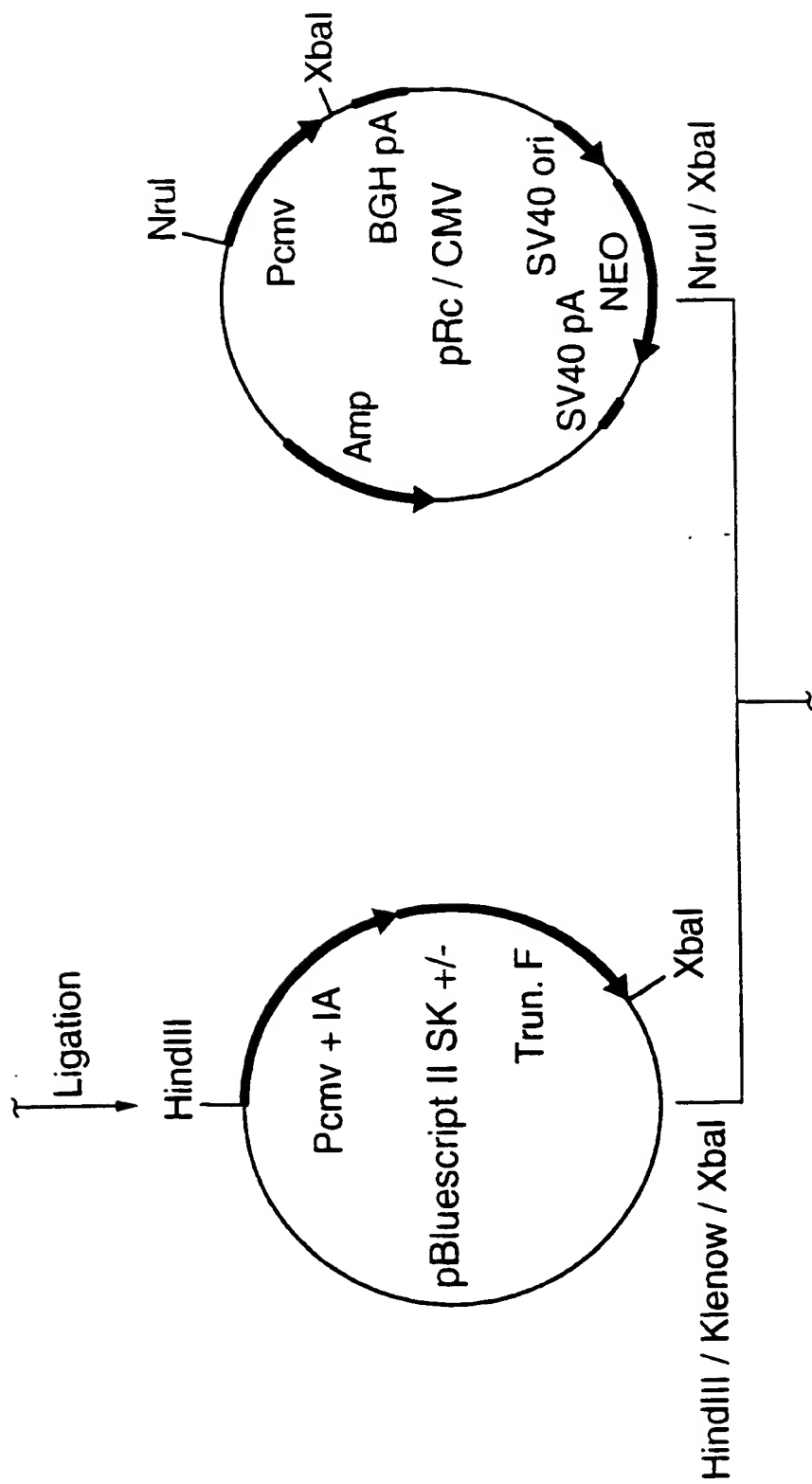


FIG.4C



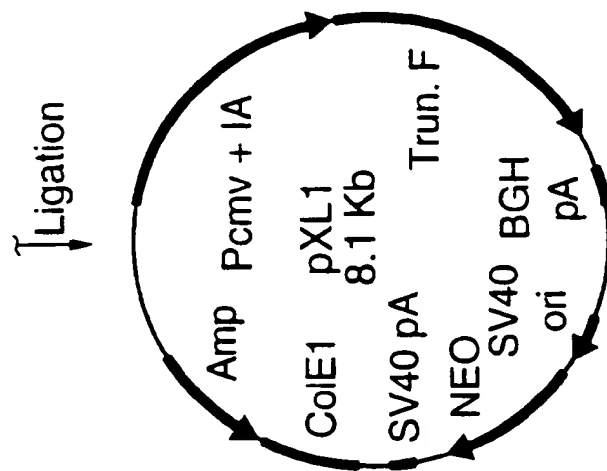


FIG.4D

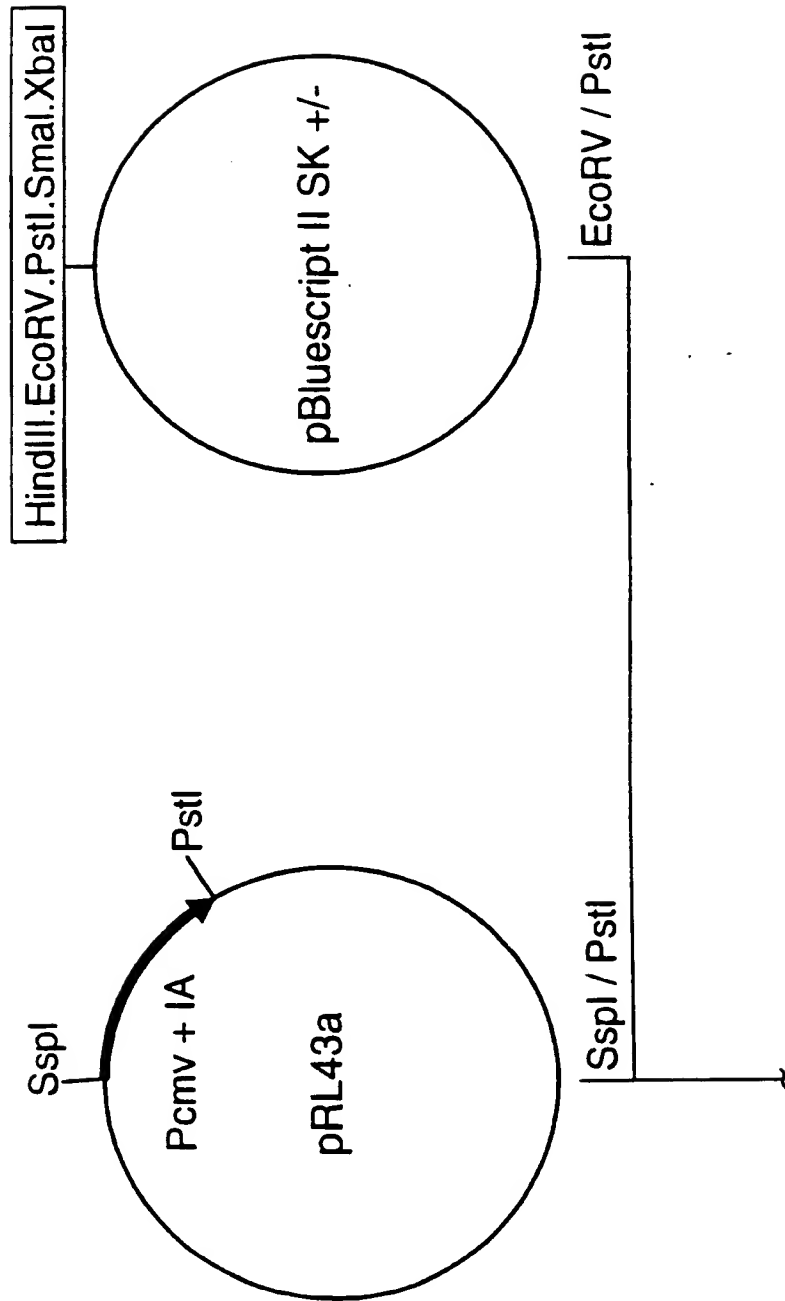


FIG.5A

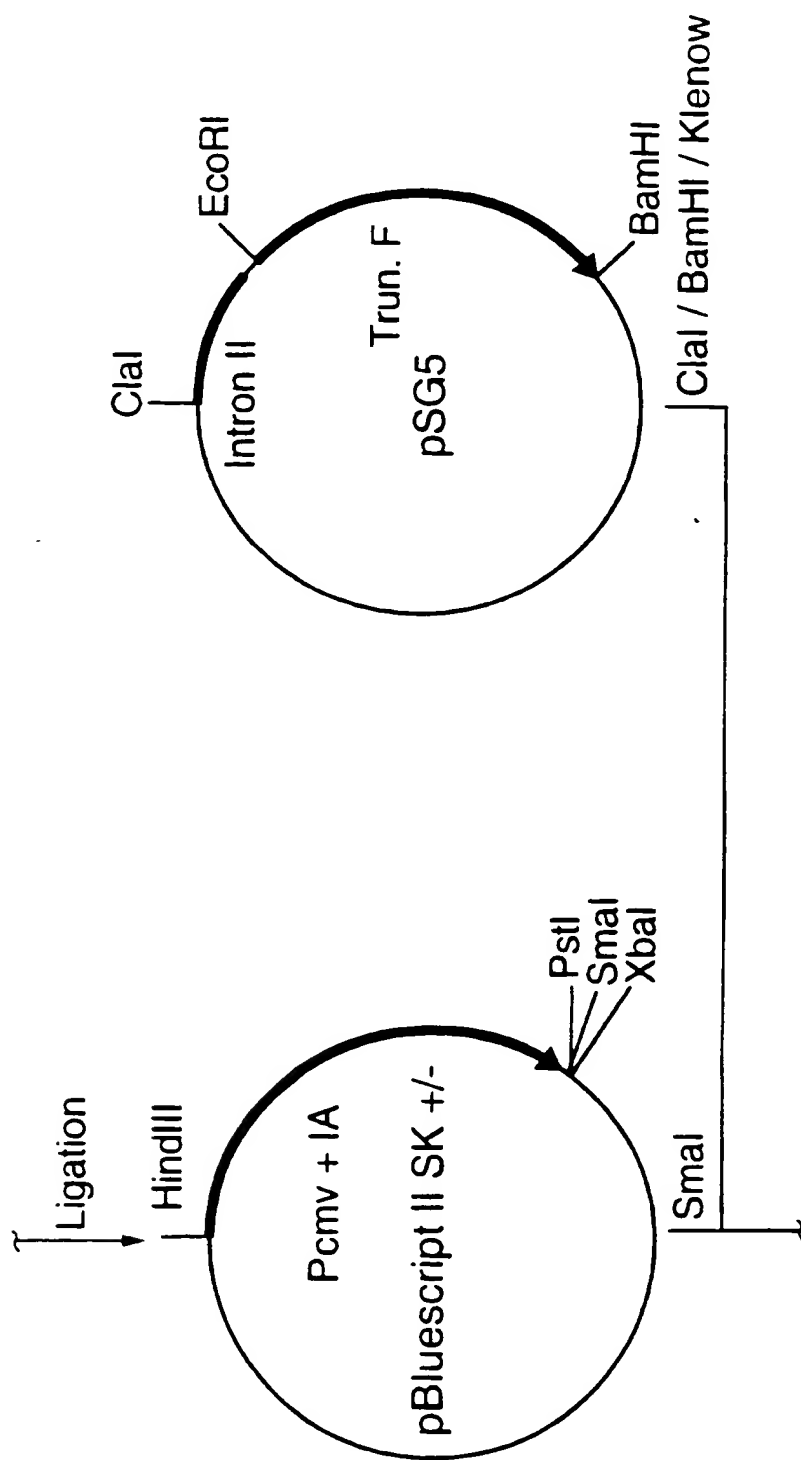


FIG.5B

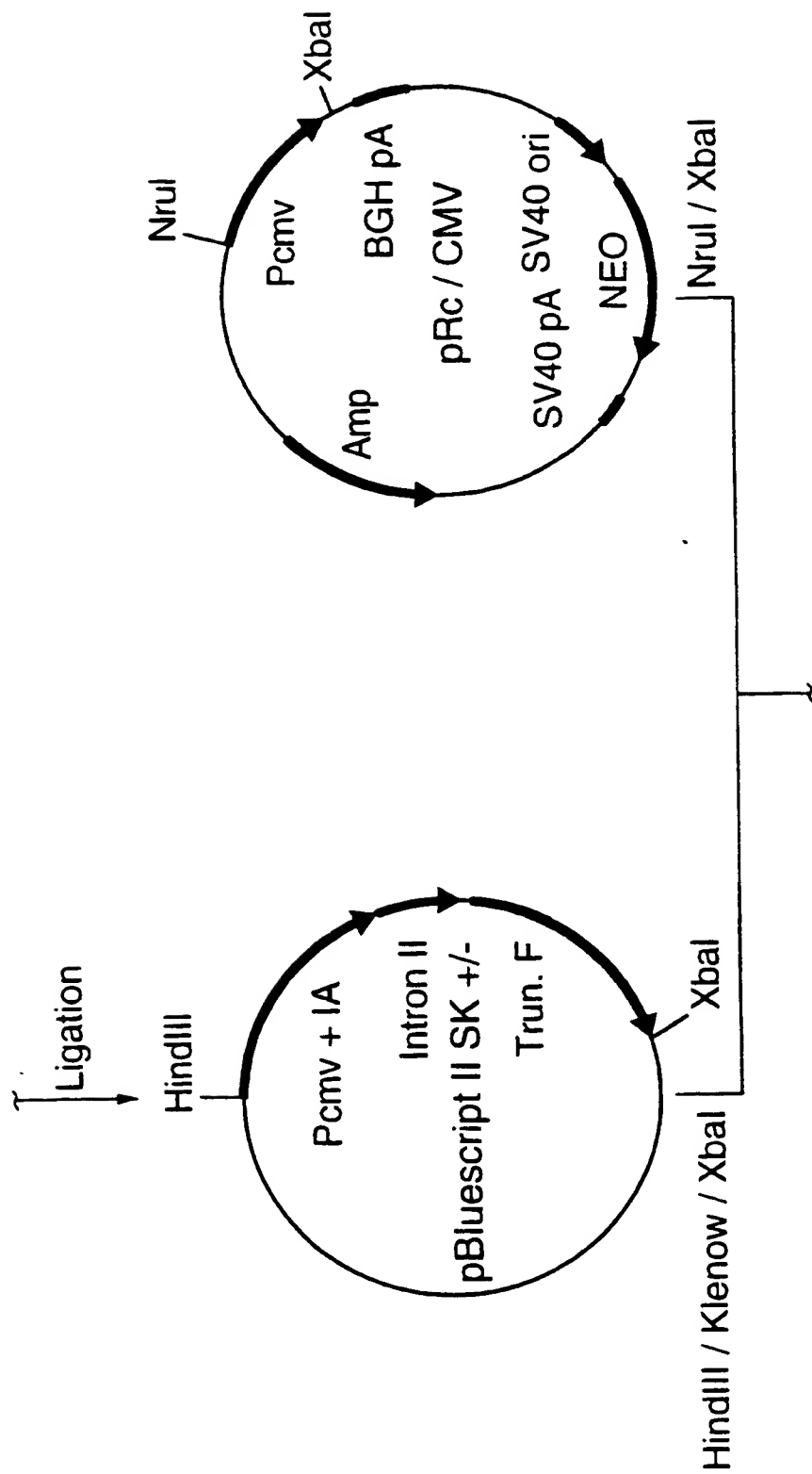


FIG.5C

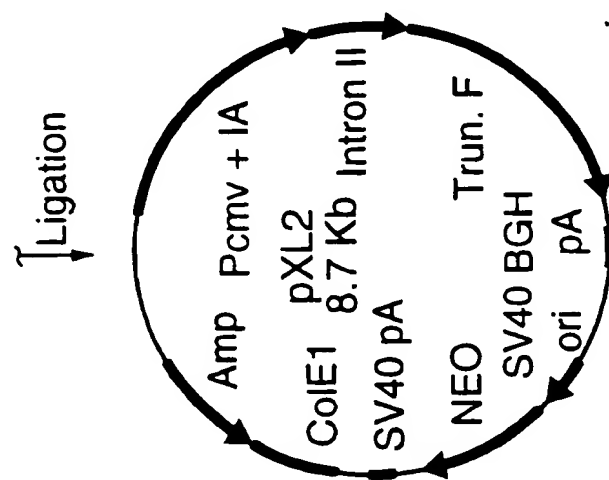


FIG.5D

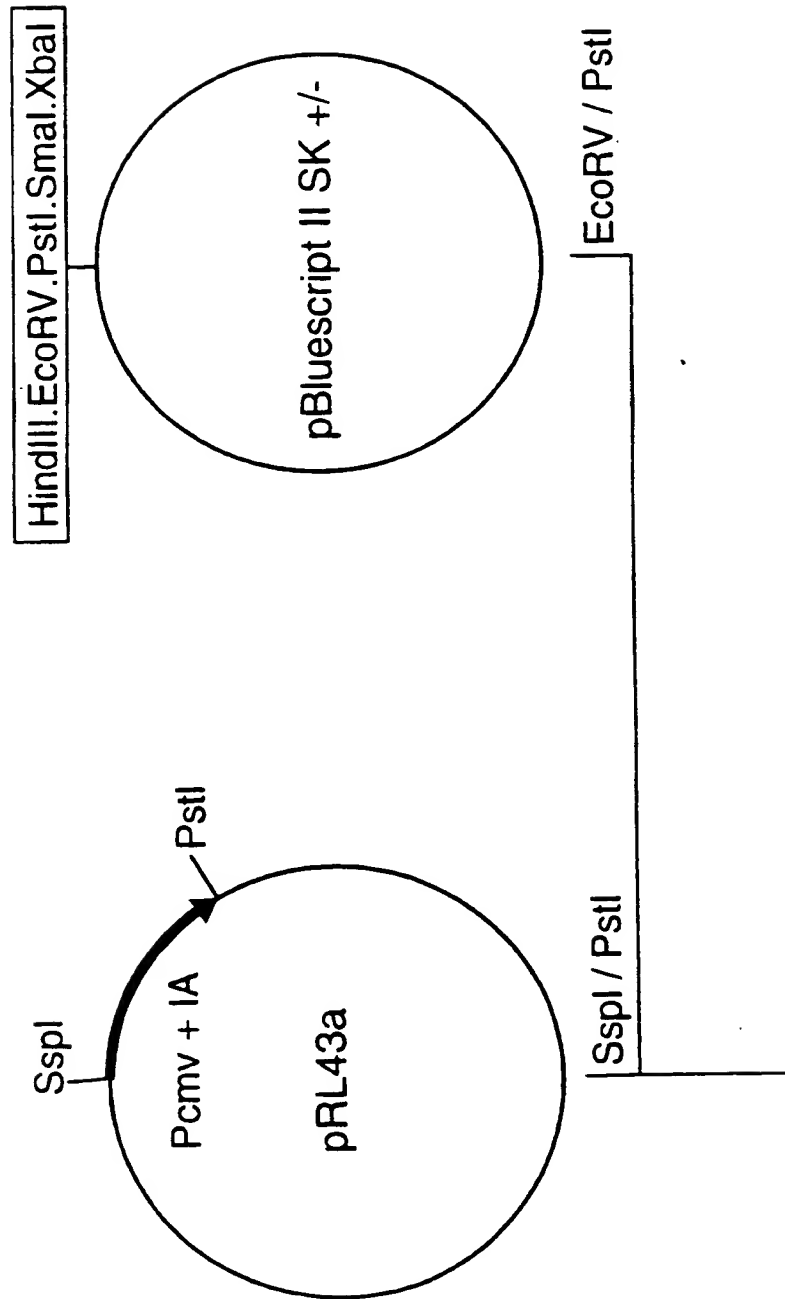


FIG.6A

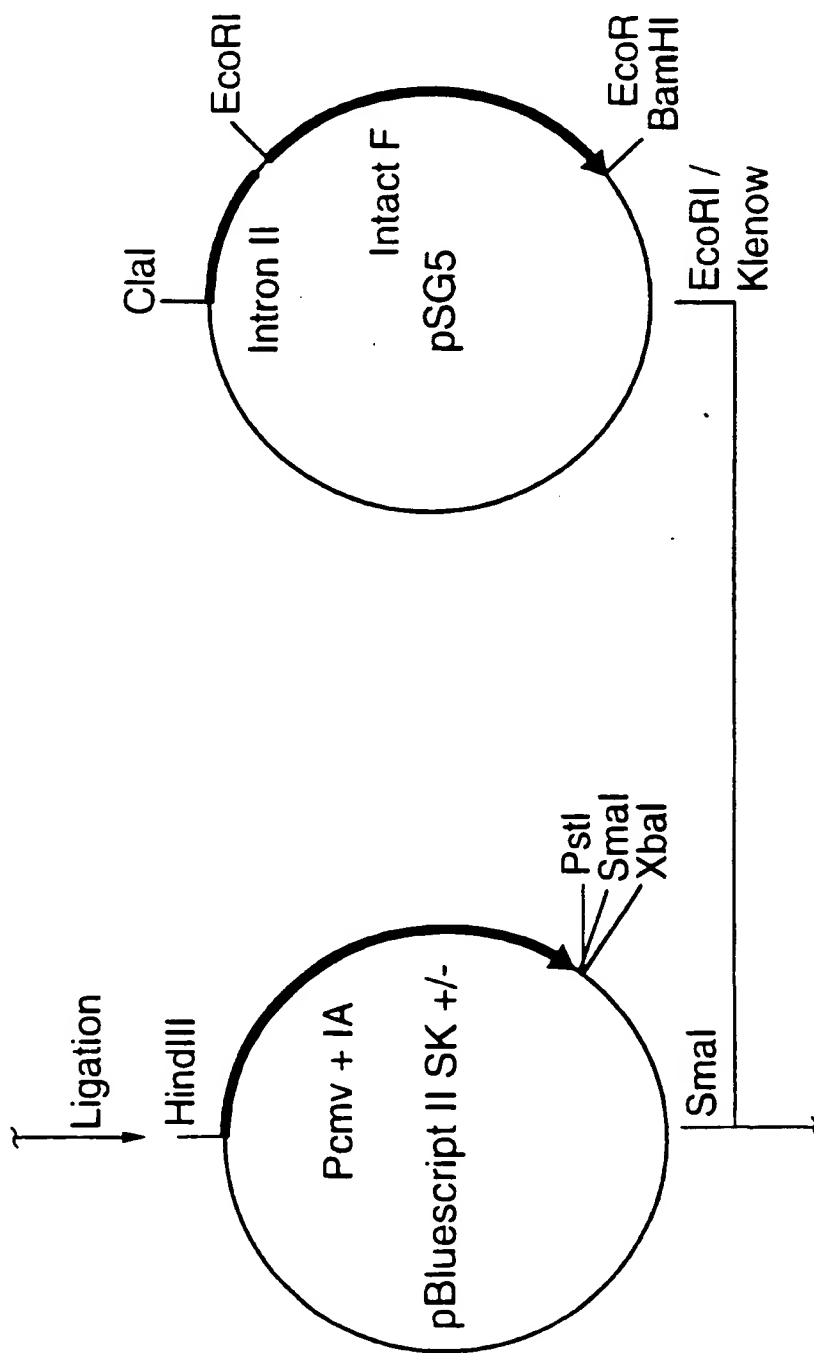


FIG.6B

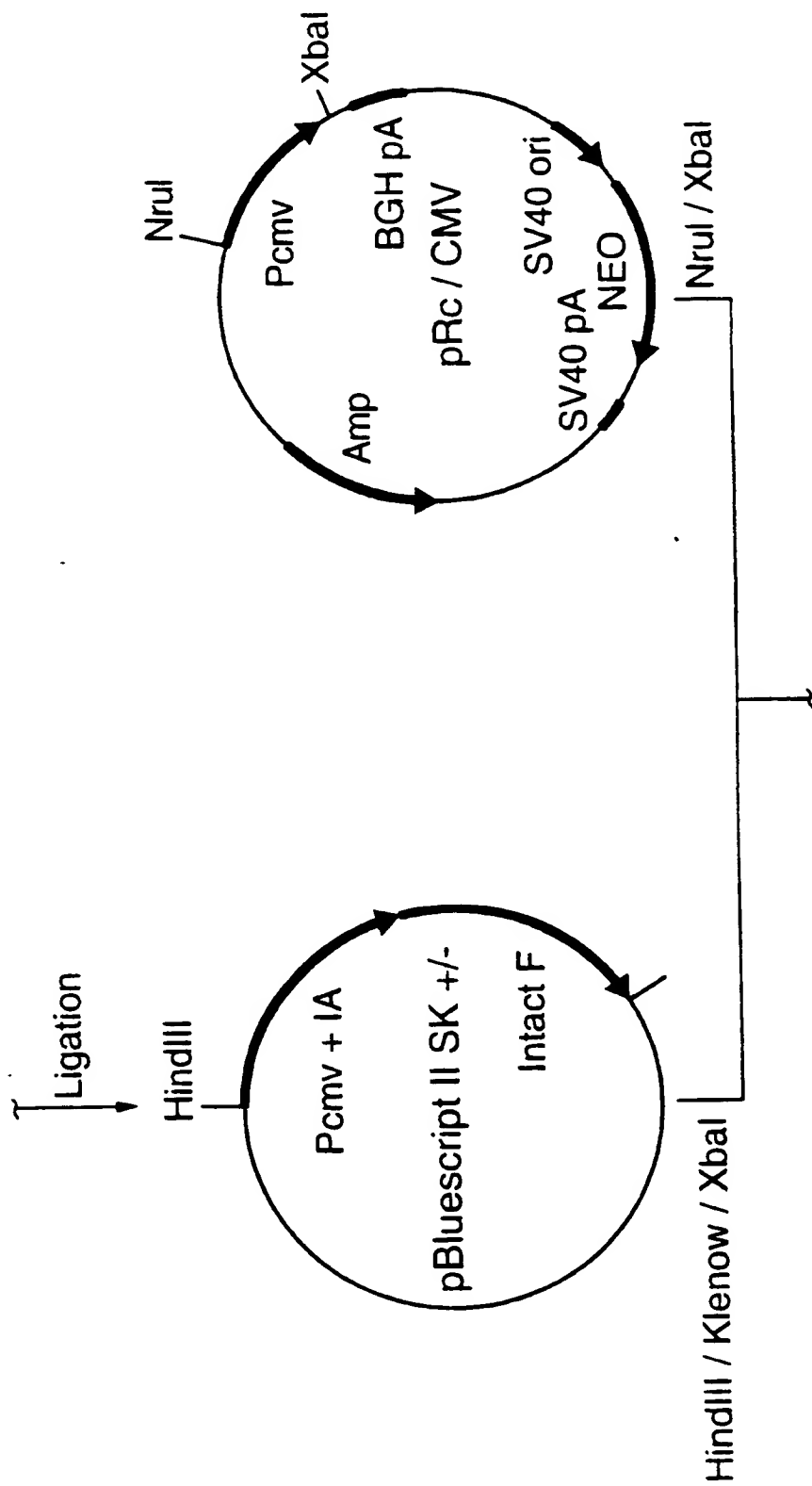


FIG.6C



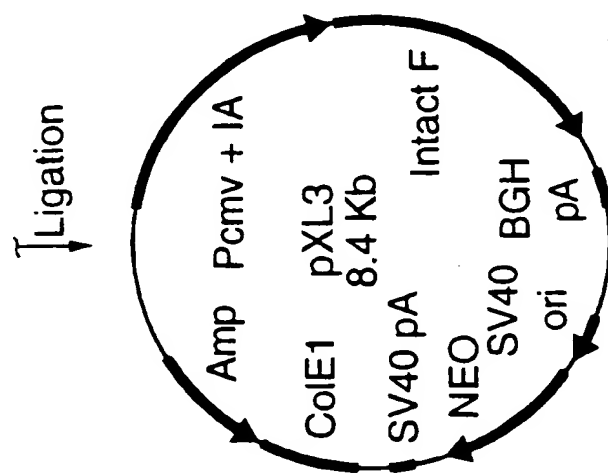


FIG.6D

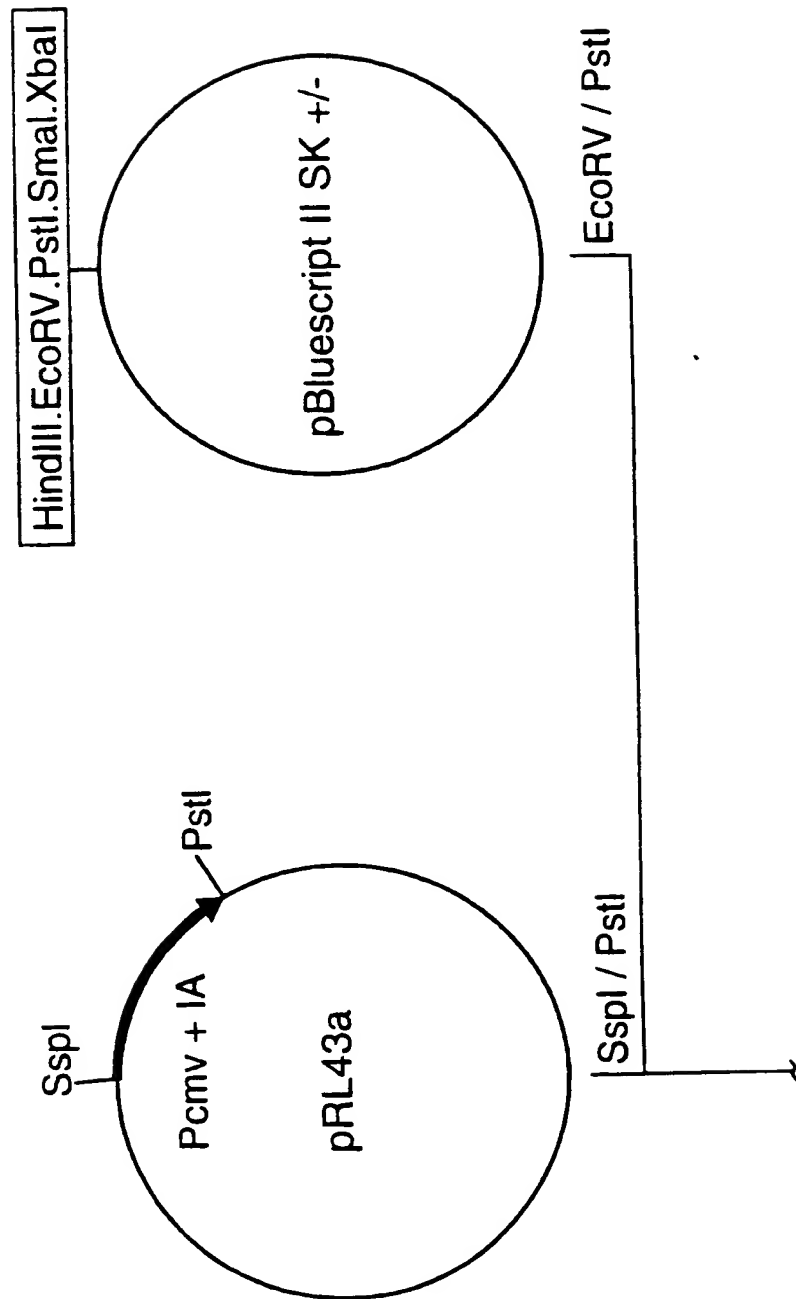


FIG.7A

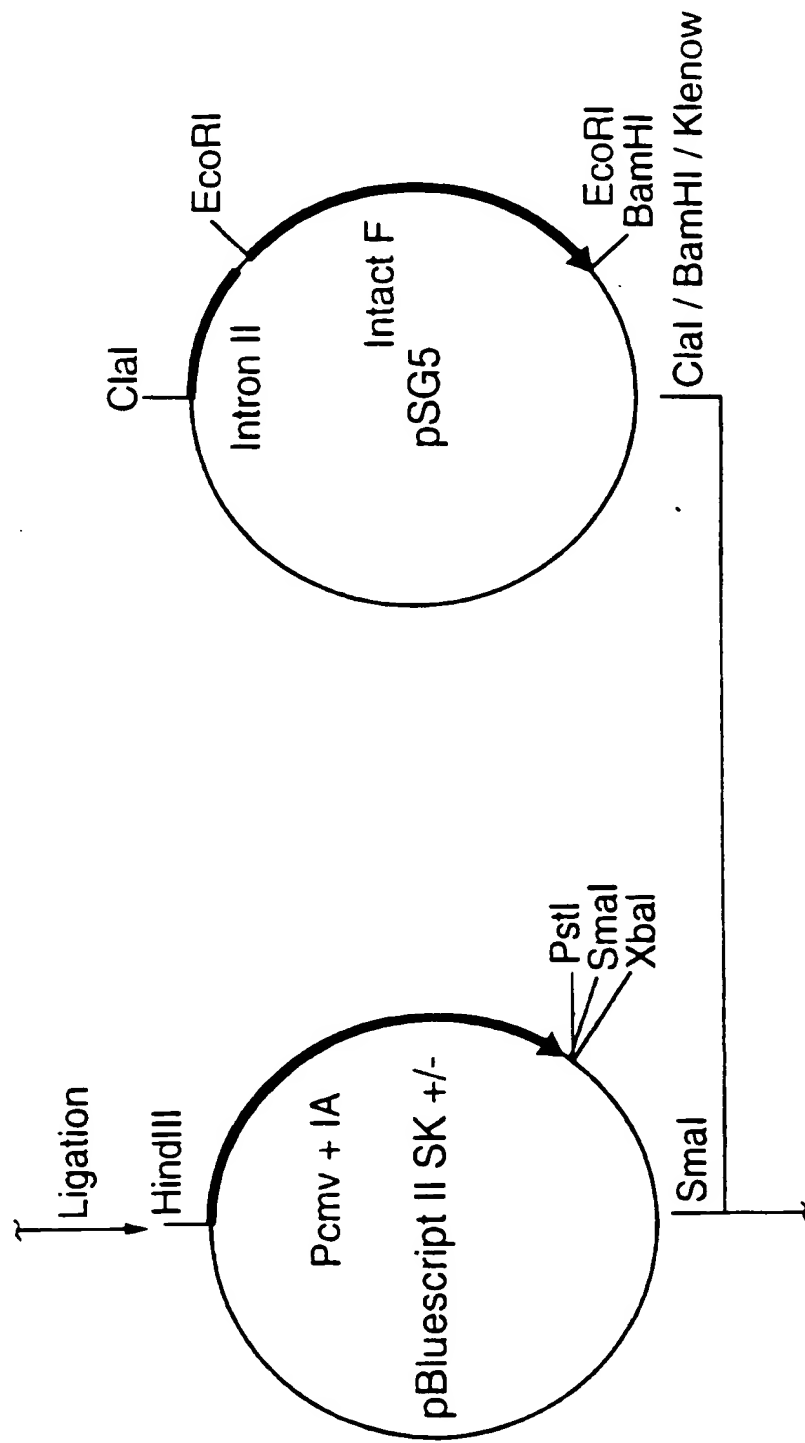


FIG.7B

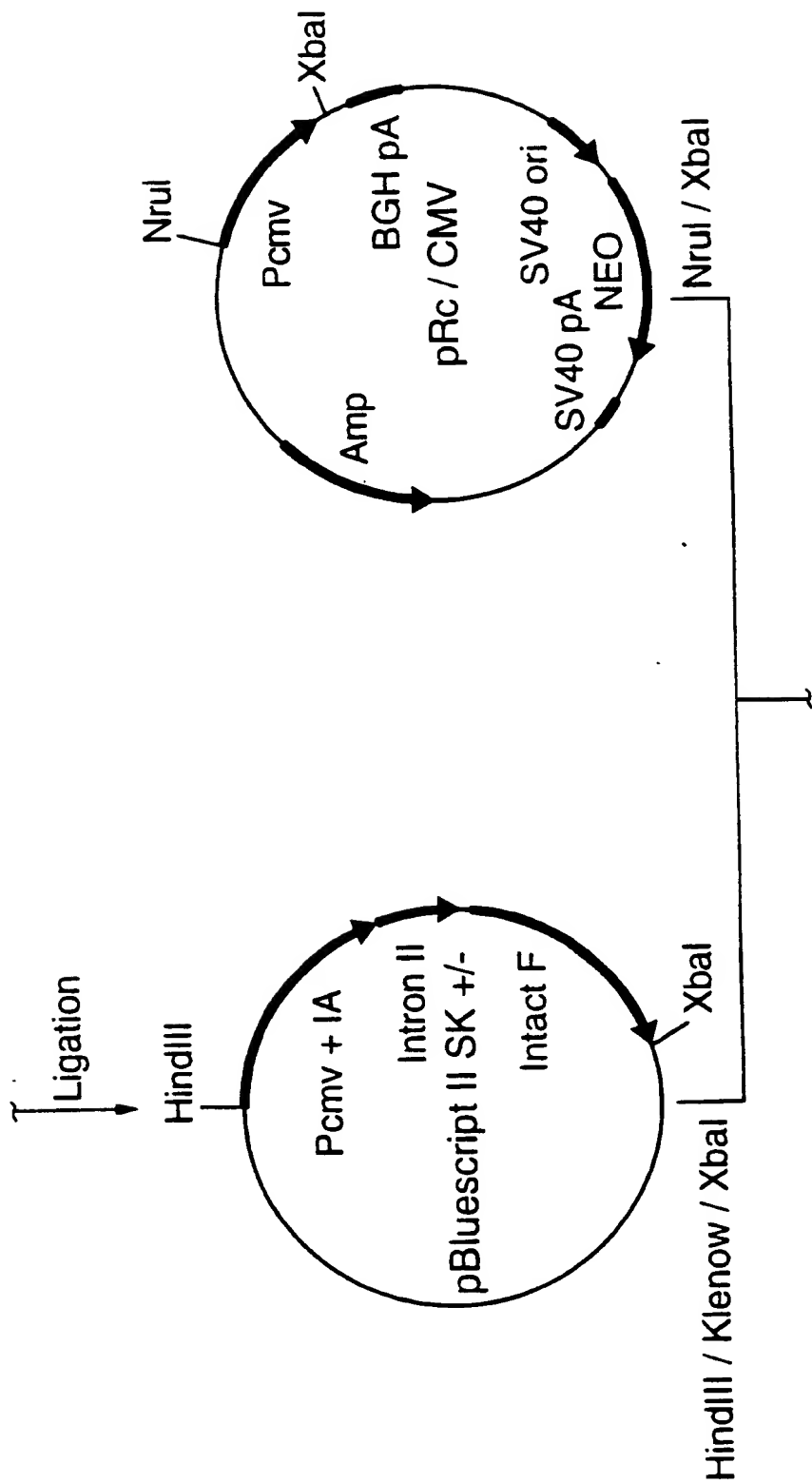


FIG.7C

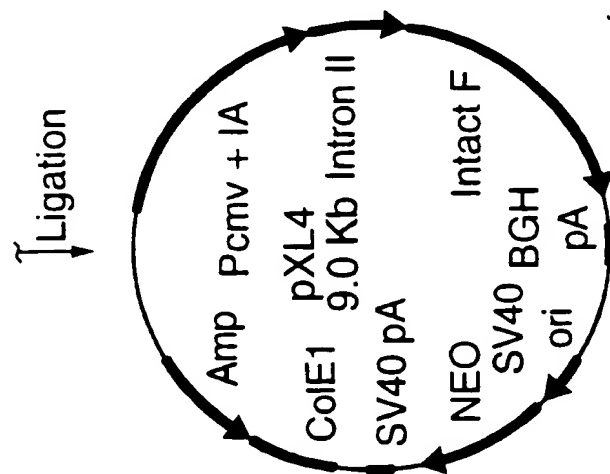


FIG.7D

FIG.8

401	TTGGGGACCC	TTGATTGTTT	TTTCTTTTTC	GCTATTGTAA	AATTCATGTT	GTGAGT
451	ATATGGAGGG	GGCAAAGTTT	TCAGGGTGTT	GTTTAGAATG	GGAAGATGTC	
501	CCTTGTATCA	CCATGGACCC	TCATGATAAT	TTTGTTTTCTT	TCACITTTCTA	
551	CTCTGTTGAC	AACCATGTC	TCCCTCTTAT	TTCTTTTTCAT	TTTCTGTAAAC	
601	TTTTTTCGTTA	AACITTAGCT	TGCATTGTGA	ACGAATTTT	AAATTCACCTT	
651	TTGTTTATTT	GTCAGATTGT	AAGTACTTTC	TCTAATCACC	TTTTTTTTCAC	
701	GGCAATCAGG	GTATATATATA	TTGTACTTCA	GCACAGTTT	AGAGAACAAT	
751	TGTTATAAAT	AAATGATAAG	GTAGAATATT	TCTGCATATA	AATCTGGCT	
801	GGCGTGGAAA	TATTCCTTATT	GGTAGAAACA	ACTACATCCT	GGTCATCATC	
851	CTGCCCTTCT	CTTATGGTT	ACAATGATAT	ACACTGTTTG	AGATGAGGAT	
901	AAAATACTCT	GAGTCCAAAC	CGGGCCCCCTC	TGCTAACCAT	GTTCATGCCT	
951	TCTTCTTTT	CCTACAG				

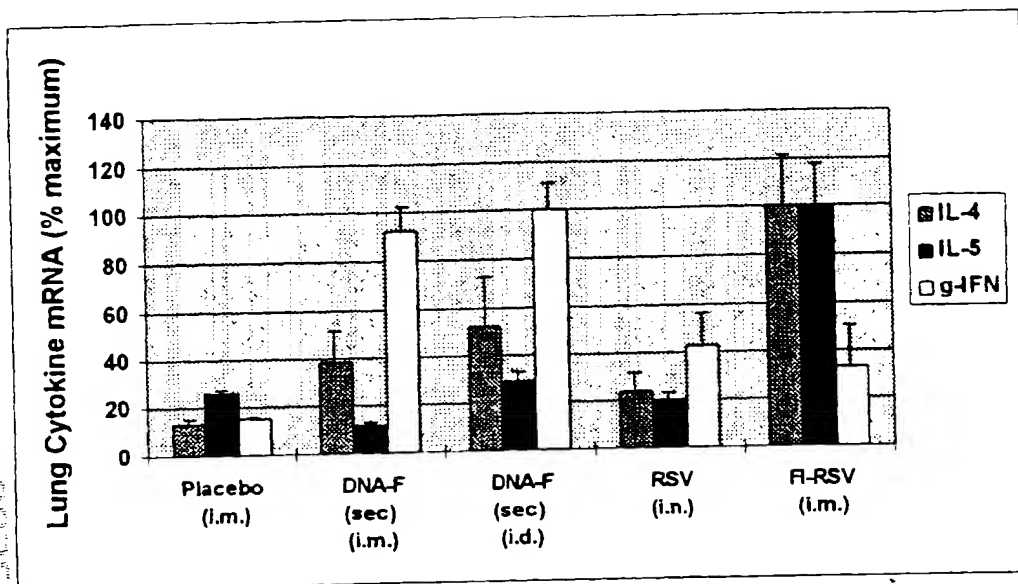


FIG 4

Figure 10 Construction of p82M35B

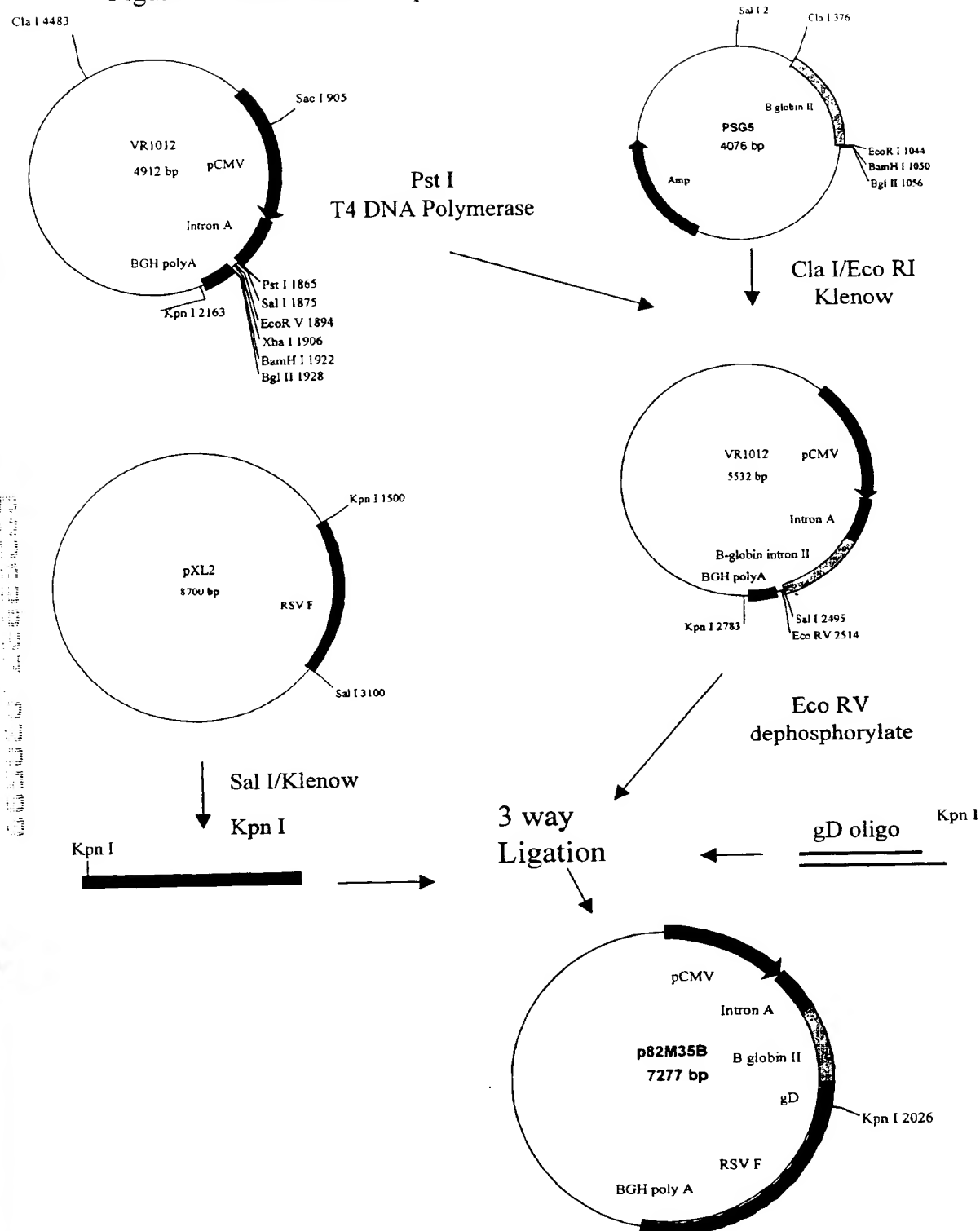




Figure 11 Nucleotide Sequence of plasmid VR1012

10	20	30	40	50	60	70
TCGCGCGTTT	CGGTGATGAC	GGTGAAAACC	TGTGACACAT	GCAGCTCCCC	GAGACGGTCA	CAGCTTGICT
80	90	100	110	120	130	140
GTAAGCGGAT	GCCGGGAGCA	GACAAGCCCC	TCAGGGCGCG	TCAGCGGGTG	TTGGCGGGTG	TCGGGGCTGG
150	160	170	180	190	200	210
CTTAAGTATG	CGGCATCAGA	GCAGATTGTA	CTGAGAGTGC	ACCATATGCG	GTGTGAAATA	CCGCACAGAT
220	230	240	250	260	270	280
GCGTAAGGAG	AAAATACCGC	ATCAGATTGG	CTATTGGCCA	TTGCATACGT	TGTATCCATA	TCATAATATG
290	300	310	320	330	340	350
TACATTTATA	TTGGCTCATG	TCCAACATTA	CCGCCATGTT	GACATTGATT	ATTGACTAGT	TATTAATAGT
360	370	380	390	400	410	420
AATCAATTAC	GGGGTCATTA	GTTCATAGCC	CATATATGGA	GTTCCGCGTT	ACATAACTTA	CGGTAAATGG
430	440	450	460	470	480	490
CCCGCCTGGC	TGACCGCCCA	ACGACCCCCG	CCCATTGACG	TCAATAATGA	CGTATGTTCC	CATAGTAACG
500	510	520	530	540	550	560
CCAATAGGGA	CTTTCCATTG	ACGTCAATGG	GTGGAGTATT	TACGGTAAAC	TGCCCCACTG	GCAGTACATC
570	580	590	600	610	620	630
AAGTGTATCA	TATGCCAAGT	ACGCCCCCTA	TTGACGTCAA	TGACGGTAAA	TGGCCCCGCT	GGCATTATGC
640	650	660	670	680	690	700
CCAGTACATG	ACCTTATGGG	ACTTTCCTAC	TTGGCAGTAC	ATCTACGTAT	TAGTCATCGC	TATTACCATG
710	720	730	740	750	760	770
GTGATGCGGT	TTTGGCAGTA	CATCAATGGG	CGTGGATAGC	GGTTTGACTC	ACGGGGATTT	CCAAGTCTCC
780	790	800	810	820	830	840
ACCCCATGTA	CGTCAATGGG	AGTTTGTITT	GGCACCAAAA	TCAACGGGAC	TTTCCAAAAT	GTCGTAACAA
850	860	870	880	890	900	910
CTCCGCCCCA	TTGACGCAAA	TGGGCGGTAG	GCGTGTACGG	TGGGAGGTCT	ATATAAGCAG	AGCTCGTTTA
920	930	940	950	960	970	980
GTGAACCGTC	AGATCGCCTG	GAGACGCCAT	CCACGCTGTT	TTGACCTCCA	TAGAAGACAC	CGGGACCGAT
990	1000	1010	1020	1030	1040	1050
CCAGCCTCCG	CGGCCGGGAA	CGGTGCATTG	GAACGCGGAT	TCCCCGTGCC	AAGAGTGACG	TAAGTACCGC
1060	1070	1080	1090	1100	1110	1120
CTATAGACTC	TATAGGCACA	CCCTTTGGC	TCTTATGCAT	GCTATACTGT	TTTTGGCTTG	GGGCCTATAC
1130	1140	1150	1160	1170	1180	1190
ACCCCGCTT	CCTTATGCTA	TAGGTGATGG	TATAGCTTAG	CCTATAGGTG	TGGGTATTG	ACCATTATTG
1200	1210	1220	1230	1240	1250	1260
ACCACTCCCC	TATTGGTGAC	GATACTTTCC	ATTACTAATC	CATAACATGG	CTCTTTGCCA	CAACTATCTC
1270	1280	1290	1300	1310	1320	1330
TATTGGCTAT	ATGCCAATAC	TCTGTCCTTC	AGAGACTGAC	ACGGACTCTG	TATTTTACA	GGATGGGGTC

1340 1350 1360 1370 1380 1390 1400  
 CCATTTATTA TTTACAAATT CACATATACA ACAACGCCGT CCCCCGTGCC CGCAGTTTTT ATTAAACATA  
 1410 1420 1430 1440 1450 1460 1470  
 GCGTGGGATC TCCACGCGAA TCTCGGTAC GTGTTCCGGA CATGGGCTCT TCTCCGGTAG CGGCGGAGCT  
 1480 1490 1500 1510 1520 1530 1540  
 TCCACATCCG AGCCCTGGTC CCATGCCTCC AGCGGCTCAT GGTCGCTCGG CAGCTCCTTG CTCCTAACAG  
 1550 1560 1570 1580 1590 1600 1610  
 TGGAGGCCAG ACTTAGGCAC AGCACAATGC CCACCACCAC CAGTGTGCCG CACAAGGCCG TGGCGGTAGG  
 1620 1630 1640 1650 1660 1670 1680  
 GTATGTGTCT GAAAATGAGC GTGGAGATTG GGCTCGCACG GCTGACGCAG ATGGAAGACT TAAGGCAGCG  
 1690 1700 1710 1720 1730 1740 1750  
 GCAGAAGAAG ATGCAGGCAG CTGAGTTGTT GTATTCTGAT AAGAGTCAGA GGTAACCTCC GTTGCGGTGC  
 1760 1770 1780 1790 1800 1810 1820  
 TGTTAACGGT GGAGGGCAGT GTAGTCTGAG CAGTACTCGT TGCTGCCCGG CGCGCCACCA GACATAATAG  
 1830 1840 1850 1860 1870 1880 1890  
 CTGACAGACT AACAGACTGT TCCTTTCCAT GGGTCTTTTC TGCAGTCACC GTCGTCGACA CGTGTGATCA  
 1900 1910 1920 1930 1940 1950 1960  
 GATATCGCGG CCGCTCTAGA CCAGGCGCCT GGATCCAGAT CTGCTGTGCC TTCTAGTTGC CAGCCATCTG  
 1970 1980 1990 2000 2010 2020 2030  
 TTGTTTGCCC CTCCCCCGTG CCTTCCTTGA CCCTGGAAGG TGCCACTCCC ACTGTCCTTT CCTAATAAAA  
 2040 2050 2060 2070 2080 2090 2100  
 TGAGGAAATT GCATCGCATT GTCTGAGTAG GTGTCAATTCT ATTCTGGGGG GTGGGGTGGG GCAGGACAGC  
 2110 2120 2130 2140 2150 2160 2170  
 AAGGGGGAGG ATTGGGAAGA CAATAGCAGG CATGCTGGGG ATGCGGTGGG CTCTATGGGT ACCCAGGTGC  
 2180 2190 2200 2210 2220 2230 2240  
 TGAAGAATTG ACCCGGTTCC TCCTGGGCCA GAAAGAAGCA GGCACATCCC CTTCTCTGTG ACACACCCTG  
 2250 2260 2270 2280 2290 2300 2310  
 TCCACGCCCC TGGTTCCTAG TTCCAGCCCC ACTCATAGGA CACTCATAGC TCAGGAGGGC TCCGCCTCA  
 2320 2330 2340 2350 2360 2370 2380  
 ATCCCAACCG CTAAAGTACT TGGAGCGGTC TCTCCCTCCC TCATCAGCCC ACCAAACCAA ACCTAGCCTC  
 2390 2400 2410 2420 2430 2440 2450  
 CAAGAGTGGG AAGAAATTAA AGCAAGATAG GCTATTAAAGT GCAGAGGGAG AGAAAATGCC TCCAACATGT  
 2460 2470 2480 2490 2500 2510 2520  
 GAGGAAGTAA TGAGAGAAAT CATAGAATTT CTCCGCTTC CTCGCTCACT GACTCGCTGC GCTCGGTCTG  
 2530 2540 2550 2560 2570 2580 2590  
 TCGGCTGCGG CGAGCGGTAT CAGCTCACTC AAAGGCGGTA ATACGTTAT CCACAGAATC AGGGGATAAC  
 2600 2610 2620 2630 2640 2650 2660  
 GCAGGAAAGA ACATGTGAGC AAAAGGCCAG CAAAAGGCCA GGAACCGTAA AAAGGCCGCG TTGCTGGCGT  
 2670 2680 2690 2700 2710 2720 2730  
 TTTTCCATAG GCTCCGCCCC CCTGACGAGC ATCACAATAA TCGACGCTCA AGTCAGAGGT GGCGAAACCC

2740	2750	2760	2770	2780	2790	2800
GACAGGACTA	TAAAGATACC	AGGCGTTTCC	CCCTGGAAGC	TCCCTCGTGC	GCTCTCCTGT	TCCGACCCCTG
2810	2820	2830	2840	2850	2860	2870
CCGCTTACCG	GATACCTGTC	CGCCTTTCTC	CCCTCGGGAA	GCGIGGCGCT	TTCTCATAGC	TCACGCTGTA
2880	2890	2900	2910	2920	2930	2940
GGTATCTCAG	TTGGGTGTAG	GTCGTTCTGCT	CCAAGCTGGG	CTGTGTGCAC	GAACCCCCCG	TTCAGCCCGA
2950	2960	2970	2980	2990	3000	3010
CCGCTGCGCC	TTATCCGGTA	ACTATCTGCT	TGAGTCCAAC	CCGGTAAGAC	ACGACTTATC	GCCACTGGCA
3020	3030	3040	3050	3060	3070	3080
GCAGCCACTG	GTAACAGGAT	TAGCAGAGCG	AGGTATGTAG	GCGGTGCTAC	AGAGTTCTTG	AAGTGGTGGC
3090	3100	3110	3120	3130	3140	3150
CTAACTACGG	CTACACTAGA	AGAACAGTAT	TTGGTATCTG	CGCTCTGCTG	AAGCCAGTTA	CCTTCGGAAA
3160	3170	3180	3190	3200	3210	3220
AAGAGTTGGT	AGCTCTTGAT	CCGGCAAACA	AACCACCGCT	GGTAGCGGTG	GTTTTTTTGT	TTGCAAGCAG
3230	3240	3250	3260	3270	3280	3290
CAGATTACGC	GCAGAAAAAA	AGGATCTCAA	GAAGATCCTT	TGATCTTTTC	TACGGGGTCT	GACGCTCAGT
3300	3310	3320	3330	3340	3350	3360
GGAACGAAAA	CTCACGTTAA	GGGATTTTGG	TCATGAGATT	ATCAAAAAGG	ATCTTCACCT	AGATCCTTTT
3370	3380	3390	3400	3410	3420	3430
AAATTAAAAA	TGAAGTTTAA	AATCAATCTA	AAGTATATAT	GAGTAACTT	GGTCTGACAG	TTACCAATGC
3440	3450	3460	3470	3480	3490	3500
TTAATCAGTG	AGGCACCTAT	CTCAGCGATC	TGTCTATTTT	GTTTCATCCAT	AGTTGCCTGA	CTCGGGGGGG
3510	3520	3530	3540	3550	3560	3570
GGGGGCGCTG	AGGTCTGCCT	CGTGAAGAAG	GTGTTGCTGA	CTCATACCAG	GCCTGAATCG	CCCCATCATC
3580	3590	3600	3610	3620	3630	3640
CAGCCAGAAA	GTGAGGGAGC	CACGGTTGAT	GAGAGCTTTG	TTGTAGGTGG	ACCAGTTGGT	GATTTTGAAC
3650	3660	3670	3680	3690	3700	3710
TTTTGCTTTG	CCACGGAACG	GTCTGCGTTG	TCGGGAAGAT	GCGTGATCTG	ATCCTTCAAC	TCAGCAAAAAG
3720	3730	3740	3750	3760	3770	3780
TTCGATTAT	TCAACAAAGC	CGCCGTCCCG	TCAAGTCAGC	GTAATGCTCT	GCCAGTGTTA	CAACCAATTA
3790	3800	3810	3820	3830	3840	3850
ACCAATTGTG	ATTAGAAAAA	CTCATCGAGC	ATCAAATGAA	ACTGCAATTT	ATTCATATCA	GGATTATCAA
3860	3870	3880	3890	3900	3910	3920
TACCATATTT	TTGAAAAAGC	CGTTTCTGTA	ATGAAGGAGA	AAACTCACCG	AGGCAGTTCC	ATAGGATGGC
3930	3940	3950	3960	3970	3980	3990
AAGATCCTGG	TATCGGTCTG	CGATTCCGAC	TCGTCCAACA	TCAATACAAC	CTATTAATTT	CCCCTCGTCA
4000	4010	4020	4030	4040	4050	4060
AAAAAAGGT	TATCAAGTGA	GAAATCACCA	TGAGTGACGA	CTGAATCCGG	TGAGAATGGC	AAAAGCTTAT
4070	4080	4090	4100	4110	4120	4130
GCATTTCTTT	CCAGACTTGT	TCAACAGGCC	AGCCATTACG	CTCGTCATCA	AAATCACTCG	CATCAACCAA

4140 4150 4160 4170 4180 4190 4200  
 ACCGTTATTC ATTCGTGATT GCGCCTGAGC GAGACGAAAT ACGCGATCGC TGTAAAAAGG ACAATTACAA  
 4210 4220 4230 4240 4250 4260 4270  
 ACAGGAATCG AATGCAACCG GCGCAGGAAC ACTGCCAGCG CATCAACAAT ATTTTCACCT GAATCAGGAT  
 4280 4290 4300 4310 4320 4330 4340  
 ATTCTTCTAA TACCTGGAAT GCTGTTTTCC CGGGGATCGC AGTGGTGAGT AACCATGCAT CATCAGGAGT  
 4350 4360 4370 4380 4390 4400 4410  
 ACGGATAAAA TGCTTGATGG TCGGAAGAGG CATAAATTCC GTCAGCCAGT TTAGTCTGAC CATCTCATCT  
 4420 4430 4440 4450 4460 4470 4480  
 GTAACATCAT TGGCAACGCT ACCTTTGCCA TGTTTCAGAA ACAACTCTGG CGCATCGGGC TTCCCATACA  
 4490 4500 4510 4520 4530 4540 4550  
 ATCGATAGAT TGTCGCACCT GATTGCCCGA CATTATCGCG AGCCCATTTA TACCCATATA AATCAGCATC  
 4560 4570 4580 4590 4600 4610 4620  
 CATGTTGGAA TTTAATCGCG GCCTCGAGCA AGACGTTTCC CGTTGAATAT GGCTCATAAC GTTCCTTGTA  
 4630 4640 4650 4660 4670 4680 4690  
 TTACTGTTTA TGTAAGCAGA CAGTTTTATT GTTCATGATG ATATATTTTT ATCTTGTCGA ATGTAACATC  
 4700 4710 4720 4730 4740 4750 4760  
 AGAGATTTTG AGACACAACG TGGCTTTCCC CCCCCCCCCA TTATTGAAGC ATTTATCAGG GTTATTGTCT  
 4770 4780 4790 4800 4810 4820 4830  
 CATGAGCGGA TACATATTTG AATGTATTTA GAAAAATAAA CAAATAGGGG TTCCGCGCAC ATTTCCCCGA  
 4840 4850 4860 4870 4880 4890 4900  
 AAAGTGCCAC CTGACGTCTA AGAAACCATT ATTATCATGA CATTAACTTA TAAAAATAGG CGTATCACGA  
 4910  
 GGCCCTTCG TC

FIGURE 12

5'AAG CTT CAG GAA CGA CCA ACT ACC CCG ATC ATC AGT TAT CCT  
TAA GGT CTC TTT TGT GTG GTG CGT TCC GGT ATG GGG GGG ACT GCC  
Met Gly Gly Thr Ala  
GCC AGG TTG GGG GCC GTG ATT TTG TTT GTC GTC ATA GTG GGC CTC  
Ala Arg Leu Gly Ala Val Ile Leu Phe Val Val Ile Val Gly Leu  
CAT GGG GTC CGC GGC AAA TAT GCC TTG GCG GAT GCC TCT CTC 3'  
His Gly Val Arg Gly Lys Tyr Ala Leu Ala Asp Ala Ser Leu

